The Role Of Serotonin In The Regulation Of Aldosterone Secretion From The Rat Adrenal Gland.

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A thesis submitted for the degree of Doctor of Philosophy, University of Edinburgh, 2000.

DECLARATION OF ORIGINALITY:

I declare that the composition of this thesis and the work presented herein is my own, unless otherwise stated, apart from the procedures listed below.

This work has not been and is not concurrently submitted for any other degree.

1. **All immunostaining procedures were carried out by Lawrence Brett of the Department of Pathology, Western general Hospital.**

2. **Urine analysis was carried out by Dick Barlow of the Western General Hospital.**

3. **Paroxetine Autoradiographs were calculated by Dr. Paul Kelly, Department of Clinical Neurosciences, Western General Hospital.**

Nicola Jane Tait Burns

DEDICATION:

I would like to dedicate this thesis to my family; Kevin, Rory, Mum, Dad, Claire and Tessa, and in memory of my Grannie. For their love, support and patience.

ACKNOWLEDGEMENTS:

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List Of Contents.

Abstract.

In 1959 Rosenkrantz *et al.*, first described the effect of the indoleamine serotonin on aldosterone secretion from the adrenal gland. To date numerous studies have reported this steroidogenic effect, describing the second messenger system utilised and in some species the receptor type involved. However the full physiological and pathophysiological role of serotonin in the control of mineralocorticoid secretion still remains to be elucidated.

Classically serotonin has always been thought to be the major indoleamine involved in the modulation of aldosterone secretion from the zona glomerulosa, a fact that has not been proven. To resolve this, numerous indoleamines, both naturally occurring and synthetic congeners, were studied in isolated rat zona glomerulosa cells for their ability to induce aldosterone secretion, as compared to serotonin. Of the compounds tested, 5-methoxytryptamine, tryptamine, N-methyltryptamine, 5-methyltryptamine, 6-methoxytryptamine and 5-hydroxytryptophan (5-HTP) stimulated, to various degrees aldosterone secretion, from the zona glomerulosa. Of those compounds tested that produced comparable stimulation of aldosterone as that produced by serotonin, cAMP output was also investigated and it was observed that all compounds stimulated cAMP output also, to varying degrees. The study allowed specific structural requirements, required for stimulation of aldosterone secretion, to be characterised, namely that the 5-hydroxyl grouping was not required for full agonist activity, but ring substitutions at other positions compromise agonist activity. The basicity of the terminal amine group is important also in receptor binding. This study will aid in the search for specific agonists and antagonists, required for the research into the serotonin receptor present within the zona glomerulosa.

Unlike the cardiovascular and CNS, where specific receptors have been identified and categorised, specific receptors within the rat zona glomerulosa for serotonin have not yet been fully characterised. The second study of this thesis aimed at characterising the receptors found within rat zona glomerulosa by utilising an array of serotonin agonists and antagonists. The antagonists ketanserin and mesulergine were found to inhibit serotonin induced aldosterone secretion, and the agonists 5-methoxytryptamine and 5-carboxamidotryptamine, produced identical stimulation of aldosterone from rat zona glomerulosa, as that produced with serotonin. This effect was also found to be affected by sodium status. These results taken together with previous studies and preliminary studies with a 5-HT_7 probe in sections of rat adrenal gland would suggest the presence of a 5-HT_7 receptor in rat zona glomerulosa, although the presence of other serotonin receptors can not be ruled out.

For serotonin to have a physiological role in aldosterone secretion, a local source of serotonin would be required. In consideration of this the role of the enzyme L-aromatic amino acid decarboxylase (L-AAAD) in the local production of serotonin and dopamine was investigated in rat adrenal zona glomerulosa, in animals maintained on varying sodium diets. The presence of L-AAAD was located, via immunohistochemistry, in the zona glomerulosa, zona fasciculata and the medulla. Conversion of 5-HTP to serotonin and L-DOPA to dopamine was observed in capsular and medullary tissue preparations, and this was inhibited by carbidopa. 5-HTP significantly stimulated aldosterone secretion, from capsular tissue. This effect was more apparent in animals on a low salt diet and less apparent in high salt diet animals as compared to animals maintained on a normal salt diet. The opposite was true for studies with L-DOPA. The effect of sodium status would suggest that the enzyme is regulated by salt intake. No significant effect of L-DOPA and of 5-HTP on corticosterone secretion from tissue preparations was observed. The results from this

study provide evidence for a role for L-AAAD in converting circulating L-DOPA and 5-HTP to dopamine and serotonin, to either inhibit or stimulate aldosterone secretion from the rat zona glomerulosa.

The role of the serotonin transporter was studied within the rat adrenal gland. The transporter molecule was visualised throughout the rat adrenal medulla via immunohistochemistry. The serotonin uptake inhibitors citalopram and desmethylimipramine (DMI) were incubated with or without serotonin in isolated rat zona glomerulosa cells and the resulting aldosterone secretion was measured. Results varied and this was thought to be due to the harsh collagenase digestion process destroying the transporter, and so whole capsular tissue was studied in a superfusion system and aldosterone secretion measured. In all cases both reuptake inhibitors increased the aldosterone output from capsular tissue. The drug of abuse methylenedioxymethamphetamine (MDMA; 'Ecstasy') was also used in this system, and this compound also caused an increase in aldosterone secretion. *In Vivo* studies with MDMA produced interesting results with an increase in plasma aldosterone and PRA. From this study evidence has been found for the existence of the serotonin transporter molecule within the rat adrenal gland. MDMA may induce a rise in aldosterone concentration within the plasma by a direct action on the serotonin transporter within the adrenal gland.

This thesis has provided strong evidence for a role of serotonin in the control of aldosterone secretion in the rat. Serotonin can be produced locally within the adrenal cortex and induce aldosterone secretion, and this action may be switched on in cases of low sodium status. Further studies are required to fully characterise the serotonin receptor, to elucidate a role for the transporter within the gland and to understand more

fully the importance of serotonin and indeed dopamine, in the physiology and pathophysiology of the adrenal cortex.

ABBREVIATIONS:

AI	-	Angiotensin I
AII	-	Angiotensin II
AIII	-	Angiotensin III
ACE	-	Angiotensin Converting Enzyme
ACTH	-	Adrenocorticotrophic Hormone
ANF	-	Atrial Natriuretic Factor
ASF	-	Aldosterone Stimulating Factor
ATP	-	Adenosine tri-phosphate
AVP	-	Arginine Vasopressin
BAL	-	British anti-lewisite
BSA	-	Bovine Serum Albumin
Ca^{2+}	-	Calcium
$CaCl_2$	-	Calcium Chloride
CAP	-	Captopril
Ci	-	Curie
CNS	-	Central Nervous System
Cpm	-	Counts Per Minute
CRH	-	Corticotrophin Releasing Hormone
cAMP	-	Adenosine 3',5'-cyclic monophosphate
cDNA	-	complementary deoxyribonucleic acid
cGMP	-	Guanosine 3',5'-cyclic monophosphate
DA	-	Dopamine
DAG	-	Sn-1,2,-diacyl glycerol
DMSO	-	Dimethyl Sulfoxide
DNA	-	Deoxyribonucleic Acid
EDTA	-	1,2-Di (2-aminoethoxy) ethane tetraacetic acid

EGTA	-	Ethyleneglycol-bis (β-aminoethyl ether)-N,N,N'-tetraacetic acid
ER	-	Endoplasmic Reticulum
EtOH	-	Ethanol
GDP	-	Guanosine di-phosphate
GTP	-	Guanosine tri-phosphate
^{3}H	-	Tritium
HCl	-	Hydrochloric Acid
5-HIAA	-	5-Hydroxyindoleacetic Acid
Hr	-	Hour
5-HT	-	5-Hydroxytryptamine
5-HTP	-	5-Hydroxytryptophan
HPLC	-	High Pressure Liquid Chromotography
^{125}-I	-	Iodinated
IP	-	Inositol mono-phosphate
IP_3	-	Inositol 1,4,5-triphosphate
K^+	-	Potassium Ion
KCl	-	Potassium Chloride
K_D	-	Dissociation Constant
KH_2PO_4	-	Potassium Dihydrogen Phosphate
K_m	-	Michaelis Constant
μ	-	Micron
μg	-	Microgram
μl	-	Microlitre
μM	-	Micromolar
M	-	Molar
ml	-	Millilitre

mg	-	Milligram
$MgSO_4$	-	Magnesium Sulphate
Min	-	Minute
mM	-	Millimolar
Na^+	-	Sodium Ion
NaCl	-	Sodium Chloride
NAD^+	-	Nicotinamide Adenine Dinucleotide
NADPH	-	Reduced Nicotinamide Adenine Dinucleotide
ng	-	Nanogram
Na_2HPO_4	-	Di-Sodium Hydrogen Phosphate
NaH_2PO_4	-	Sodium Di-Hydrogen Phosphate
NaOH	-	Sodium Hydroxide
NaN_3	-	Sodium Azide
nM	-	Nanomolar
PI	-	Phosphatidylinositol
PIP_2	-	Phosphatidylinositol 4,5-biphosphate
pM	-	Picomolar
PRA	-	Plasma Renin Activity
RAS	-	Renin-Angiotensin System
RIA	-	Radioimmunoassay
RNA	-	Ribonucleic Acid
SEM	-	Standard Error of Mean
V/V	-	Volume for Volume
W/V	-	Weight for Volume
W/W	-	Weight for Weight

ZF	-	Zona Fasciculata
ZG	-	Zona Glomerulosa
ZR	-	Zona Reticularis

LIST OF PUBLICATIONS ARISING FROM THIS THESIS.

Abstracts.

1) Burns, N., Hughes, M., Williams, B.C. & Olverman, H.J. (1994). Effects of indoleamines on aldosterone secretion from rat zona glomerulosa cells. *Br. J. Pharmacol. Proc. Supp.*, **111,** 111P.

2) Burns, N., Hughes, M., Olverman, H.J.O. & Williams, B.C. (1994). Indoleamine-stimulated aldosterone secretion – Structure activity relationships. *J. Endoc. Supp.*, **143**, 104P.

3) Burns, N., Sephton, C., Brett, L., Olverman, H.J. & Williams, B.C. (1995). Autoradiographic and immunohistochemical localisation of 5-HT transporter sites in rat adrenal. *Br. J. Pharmacol. Proc. Supp.*, **116**, 224P.

4) Burns, N., Sephton, C., Brett, L., Olverman, H.J.O., Lee, M.R. & Williams, B.C. (1996). The role of L-aromatic amino acid decarboxylase in serotonin stimulated aldosterone secretion in response to salt intake. *J. Endoc. Supp.*, **148**, 79P.

5) Burns, N., Olverman, H.J.O., Williams, B.C.W. & Kelly, P. (1996). In vivo and in vitro effects of MDMA on aldosterone secretion. *J. Endoc. Supp.*, **148**, 77P.

6) Burns, N., Brett, L., Kelly, P.A.T., Lawrence, J.A., Olverman, H. J. & Williams, B.C. (1996). Specific localisation of the 5-HT transporter in the rat adrenal medulla. *Br. J. Pharmacol. Proc. Supp.*,**117**, 91P.

7) Burns, N., Sephton, C., Brett, L., Olverman, H.J. & Williams, B.C. (1996) Role of L-aromatic amino acid decarboxylase in the regulation of aldosterone secretion by 5-HT. *Br. J. Pharmacol. Proc. Supp.*,**117**, 267P.

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Papers.

1 Burns, N., Olverman, H.J., Kelly, P.A.T. & Williams, B.C. (1996). Effects of ecstasy on aldosterone secretion in the rat *in vivo* and *in vitro*. *Endoc. Res.*, **22**, 601-606.

Chapter One

The Adrenal Gland and the Regulation of Aldosterone Secretion.

1.1. Introduction.

This chapter will focus on the adrenal gland, the zonation and interactions between the various zones of the gland, and in particular the steroid hormone aldosterone will be discussed, focussing on the biosynthesis and factors controlling the secretion of this hormone. Though various factors have been reported to affect aldosterone secretion, only those factors whose physiological roles are established or are relevant to the studies carried out will be discussed.

1.2. Embryology, Development and Blood Supply of the Adrenal Gland.

The adrenal glands lie in the abdominal cavity, close to the upper pole of the kidneys, surrounded by adipose tissue and by renal fasciae to which they firmly adhere. The glands themselves are divided into two functionally distinct areas, the outer steroid-synthesising cortex and the inner catecholamine-producing medulla, which are of different embryological origin. In the developing adrenal gland the adrenocortical cells arise from the mesoderm, in particular from the columnar epithelial cells lining the coelom, chromaffin cells arise from the paraganglion cells of the neural crest complex which invade the presumptive cortical cell mass and eventually become surrounded by the mesodermal cells to form the adrenal medulla. The ectodermal cells of the adrenal medulla then differentiate to form either mature ganglionic cells or the chromaffin cells that secrete catecholamines.

The blood supply to the adrenal glands originates from small arteries branching from the aorta (at the level of the superior mesenteric artery) and from the inferior phrenic and renal arteries (Dobbie *et al.*, 1968). These three main vessels break up as they approach the gland into arteriae comitantes and penetrate the capsule of the gland to form an extensive subcapsular arterial plexus. In the rat, the subcapsular arterioles have muscular walls suggesting that they may be a site for the control of blood flow through the gland. Effluent blood is collected into a single central vein

emerging from the anterior surface of the gland. This vein is engulfed by a cuff of cortical tissue, which exists at the hilus of the suprarenal vein. Only in the head region of the gland do small muscular venous radicals lie free in the medullary tissue, unencompassed by this cortical sleeve. In the human glands, this vein discharges either directly into the superior vena cava, as in the right gland, or joins the inferior phrenic vein, before entering the renal vein, as in the left gland. In the rat, the central vein is very thin as it traverses the gland, and becomes thicker as it is exteriorised.

Inside the gland arterial blood flows through capillary loops which surround the cortical cells of the zona glomerulosa (ZG). These capillaries widen and descend through the ZG to the zona fasciculata (ZF), and once they reach the zona reticularis (ZR) they form the plexus reticularis. The blood now either drains from this plexus into the sinusoides of the alar raphe, or, in regions containing medullary tissue, the blood flows in to the medulla itself. This cortico-medullary portal system is thought to be the sole source of medullary blood in the rat and bovine adrenal (Vinson *et al.*, 1985), although the human medulla is also supplied directly by the arteriea medullae. The deeper cortical layers thus have no direct arterial supply, but receive blood that has already perfused the outer cell layers. This centripetal arrangement is extremely well preserved between species, and although the size of the gland may alter from species to species, the thickness of the cortex remains the same due to this centripetal arrangement. In order to maintain adequate perfusion of the deeper cortical layers in larger mammals, adrenal bulk is increased by lateral expansion and convolution, rather than increases in cortical width, as an increase in cortical width would cause the venous end of the capillary bed to be too far from the arterial supply. The centripetal blood supply has important implications in the gradient theory of adrenocortical zonation, and the vasculature itself plays an important role in cortico-medullary interactions.

1.3. Adrenocortical Zonation.

In 1858 Harley first described the different zones of the adrenal cortex, describing the three concentric shells, which were then named in 1866 by Arnold as the ZG (glomus-ball), the ZF (fascis-bundle) and the ZR (rete-net). Originally these divisions were made on the basis of morphological differences between the cells within each shell, but it is now evident that the adrenocortical zones are functionally distinct. Different enzymes in each zone lead to differences in the steroids secreted by them. Thus the hormone aldosterone is secreted exclusively by the ZG, whereas the inner ZFR produces mainly cortisol (in primates) and corticosterone (in rodents). Androgens are secreted by the ZR and the inner zones of the foetal adrenal. Figure 1.1. depicts a diagrammatic representation of the adrenal cortex.

The outermost zone, the ZG, lies just below the connective tissue capsule and forms the periphery of the cortex. This zone accounts for ~ 15% of the total mass of the cortex and comprises relatively small cells arranged in rounded ball-like clusters with a high nuclear/cytoplasmic ratio. The zone is characterised by transverse foldings of the cristae of the mitochondria. The cytoplasm is relatively low in lipid. The intermediate ZF comprises the bulk of the cortex, ~ 70%, and consists of relatively large cells arranged in radially disposed columnar layers extending from the ZG. These cells are characterised by their low nuclear/cytoplasm ratio, lipid-rich cytoplasm and small ovoid mitochondria with tubulovesicular cristae. They are described as "clear cells", and are separated by venous spaces, reticular fibres and sinusoidal capillaries. The rest of the cortex, ~ 20%, comprises the innermost zone, the ZR, lying adjacent to the medulla. The cells in this zone are of intermediate size, often referred to as "compact cells", and exhibit a network arrangement. They have an eosinophilic cytoplasm with few mitochondria and lipid droplets. The cells are easily characterised by their pigmentation due to the presence of lipofuscin granules. The cortex surrounds the adrenal medulla which is composed of columnar shaped chromaffin cells,

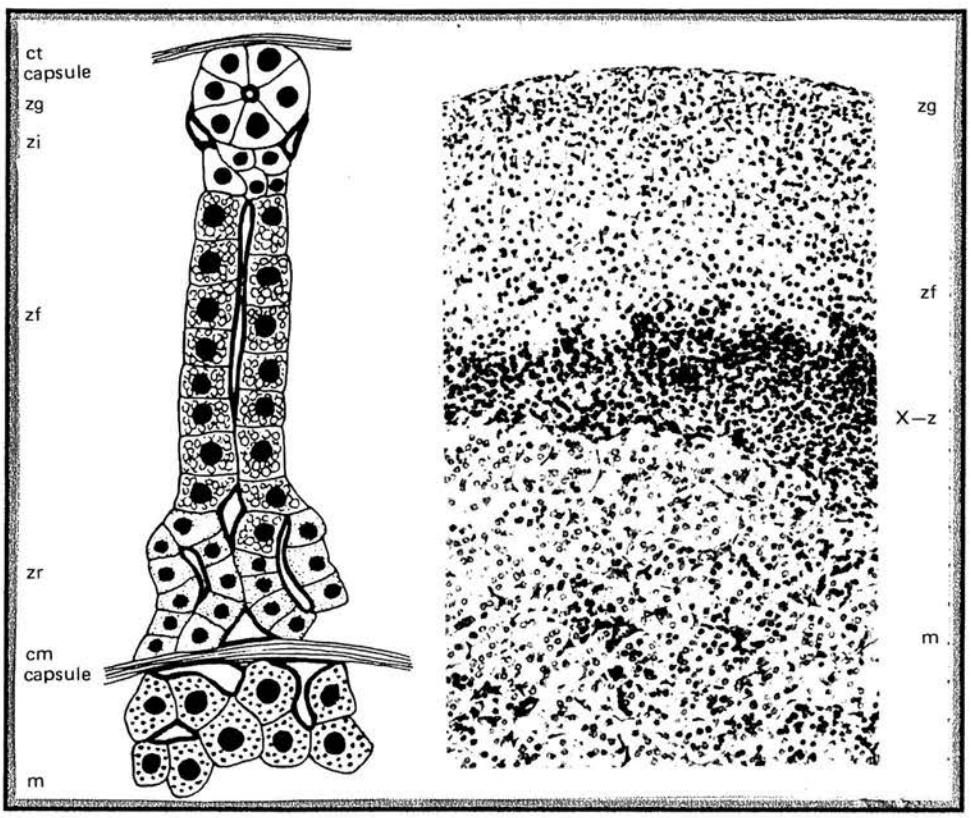


Figure 1.1. Diagrammatic representation of adrenal gland histology (From Gorbman, A. & Bern, H.A. (1962) A textbook of comparative endocrinology, Wiley, New York, London).

individually innervated by cholinergic preganglionic neurones and full of membrane bound electron dense granules that are thought to be the main storage sites of the catecholamines noradrenaline and adrenaline.

The exact cellular arrangement of the cortex varies from species to species. In the human adrenal gland the ZG and the ZF merge imperceptibly with ZF cells often observed directly underneath the capsule. In contrast, the ZG of the bovine adrenal gland is sharply defined from the inner zones, while the ZF and ZR form a single uniform layer. The three zones are clearly distinguishable in the rat, guinea-pig and rabbit cortex, while the amphibian gland displays no significant zonation.

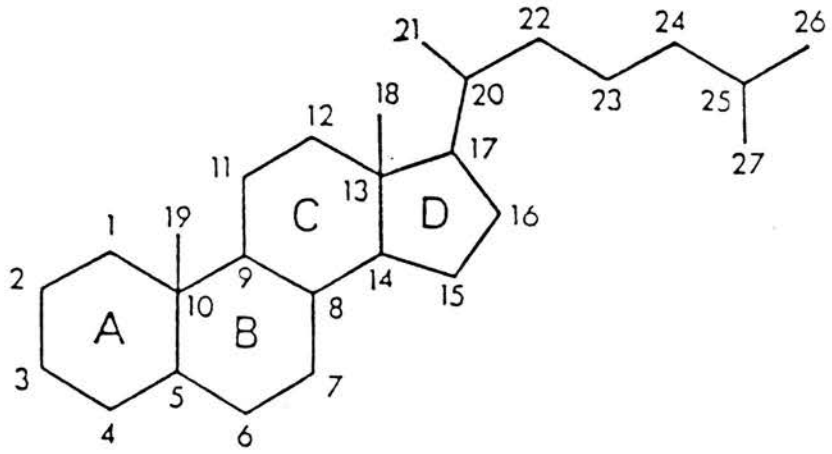
Other adrenocortical zones have been described in various species. In 1957 Chester-Jones, *et al.*, described a zone, lying between the ZR and the medulla, in the adrenals of young mice and named it the X-zone. This zone disappears following sexual maturity in the male and after the first pregnancy in the female, leaving behind a band of connective tissue. However in the male rat the X-zone forms the ZR in adult life (Shire & Stewart, 1972). This zone seems to play a role in foetal or post-natal life. In rat, rabbit, cat, dog, cattle and sheep a fourth zone is present termed the zona intermediate (ZI), lying between the ZG and the ZF (Deane, 1962). The human and primate foetal adrenal cortex exhibits two zones, the outer definitive and the inner foetal zone. Also, a special zone, found only in the inner cortex of adult female marsupials.

The cells from these different zones within the adrenal cortex, represent different phenotypes of a single cell type. The adult adrenal cortex undergoes permanent regeneration. The maintenance of normal adrenocortical size involves cell proliferation in the ZG, subsequent centripetal displacement and differentiation in the ZF and cell deletion through apoptosis in the deep fasciculata and ZR zones (Belloni *et al.*, 1978; Wyllie *et al.*, 1973; Orth *et al.*, 1992).

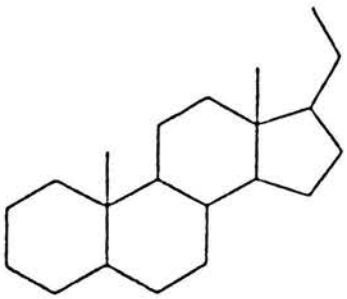
1.4. Adrenocortical Steroids

All steroids have the same basic four ringed structure and a various number of carbon atoms from which a number of substitutions, for example ketones, aldehydes and hydroxyls, may extend above (β) or below (α) the plane of the central ring structure. The biological properties of a steroid are determined by the number and structure of the functional groups attached to the basic steroid nucleus. There are four parent hydrocarbon molecules on which all the major steroids are structurally based (Figure 1.2.). The first, cholestane (C27), which may be considered chemically to be the parent compound for cholesterol and its related metabolites which include the bile acids (C24). The second, pregnane (C21), is the parent hydrocarbon for a group of steroids which are characteristic of the adrenal cortex. These include the glucocorticoids corticosterone and cortisol and the mineralocorticoid aldosterone. The third, androstane (C19) has no side chain on ring D and this group includes compounds such as androstenedione and testosterone. The last parent molecule, estrane (C18), includes the phenolic steroids such as estradiol-17ß and estriol.

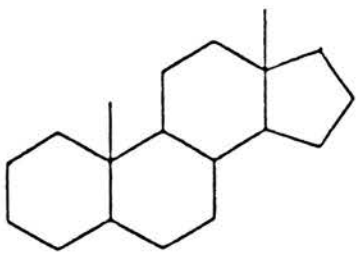
The major adrenal steroids secreted are those with mineralocorticoid or glucocorticoid activity, and the sex steroids. The major mineralocorticoid produced by the adrenal cortex is aldosterone, a 21 carbon molecule with an 11β-hydroxyl group. Aldosterone usually exists in an hemiacetal form with the aldehyde group at C18. The formation of aldosterone is dependent on the presence of the enzyme P450aldo (aldosterone synthase)(Figure 1.3.). This enzyme is only present within the ZG. Glucocorticoids are produced primarily by the ZF and ZR. The major glucocorticoid produced by human, bovine and sheep is cortisol. The presence of the enzyme P450c17 (17α-hydroxylase), is a requirement for cortisol production. Most rodent adrenals do not have this enzyme, and so corticosterone is the major glucocorticoid secreted in these species. The main adrenal androgens,



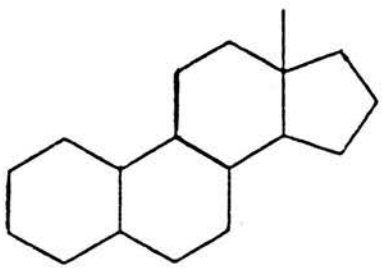
Cholestane



Pregnane



Androstane



Estrane

Figure 1.2. Basic structures of the parent hydrocarbon molecules, pregnane (C21), androstane (C19), estrane (C18) and cholestane (C27).

androstenedione, DHEA and 11β-hydroxyandrostenedione, are secreted predominantly by the ZR in humans and guinea-pigs, but by both the ZF and ZR in rats.

1.5. Adrenocorticol Steroid Biosynthesis

All of the steroids synthesised in the adrenal cortex are derived from cholesterol, which can either be synthesised within the adrenal cortex and in other tissues, from acetate, or derived from animal fat in the diet. Cholesterol is stored, as cholesteryl ester droplets, within the cell. Stimulation of steroidogenesis, leads to the conversion of cholesterol esters to free cholesterol, which is then transferred from the outer to the inner mitochondrial membrane by steroidogenic acute regultory protein (StAR), where conversion to the 21-hydrocarbon, pregnenolone takes place (Figure 1.3; Figure 1.4). Pregnenolone is then transported from the inner mitochondrial membrane to the endoplasmic reticulum by another binding protein. In the endoplasmic reticulum, pregnenolone is converted to progesterone, from here depending on the tissue, progesterone can enter a number of biosynthetic pathways catalysed by enzymatic cascades, producing a range of steroid hormones with various biological activities. For example within the adrenal cortex progesterone is hydroxylated to 11-deoxycorticosterone, which is then transferred from the microsomes back to the mitochondria for conversion to corticosterone. Corticosterone is converted to aldosterone via 18-hydroxycorticosterone, alternatively 11-deoxycorticosterone can be hydroxylated to 18-hydroxydeoxycorticosterone before being hydroxylated to 18-hydroxycorticosterone (Vinson *et al.*, 1991).

Steroid hormone output is regulated at a number of levels. Substrate availability is a major regulatory factor, especially the rate of cholesterol transport to the inner mitochondrial membrane. Enzyme activity and mass of the zone in which the steroid is synthesised are also important factors. The levels of the steroidogenic

ENZYMES	REACTIONS	PRODUCTS
		CHOLESTEROL
P450scc	22R-Hydroxylation 20α-Hydroxylation Side-Chain-Splitting	↓
		PREGNENOLONE
3β-HSD	3β-Hydroxydehydro-genation Transfer of double bond ($\Delta^5 \rightarrow \Delta^4$)	↓
		PROGESTERONE
P450c21	21-Hydroxylation	↓
		11-DEOXYCORTI-COSTERONE
P450aldo	11β-Hydroxylation	↓
		CORTICOSTERONE
P450aldo	Methyl oxidation 1 (18-Hydroxylation)	↓
		18-HYDROXYCORTI-COSTERONE
P450aldo	Methyl oxidation 2 ("18-Hydroxydehydro-genation")	↓ ↓ ↓ ↓
		ALDOSTERONE

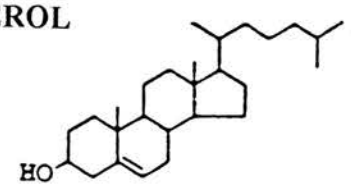
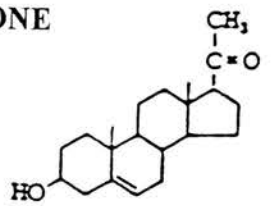
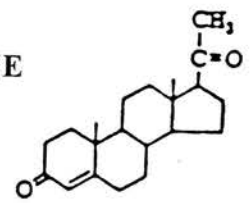
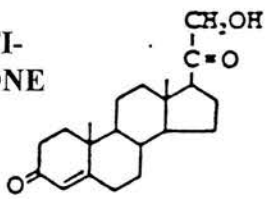
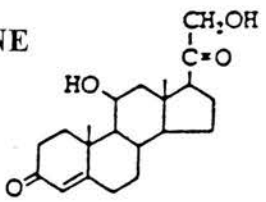
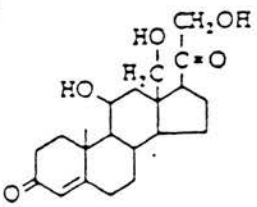
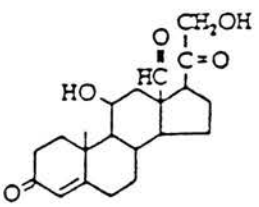








Figure 1.3. Diagram depicting the major steroid interconversions within the adrenal cortex of rats in the formation of aldosterone from cholesterol.

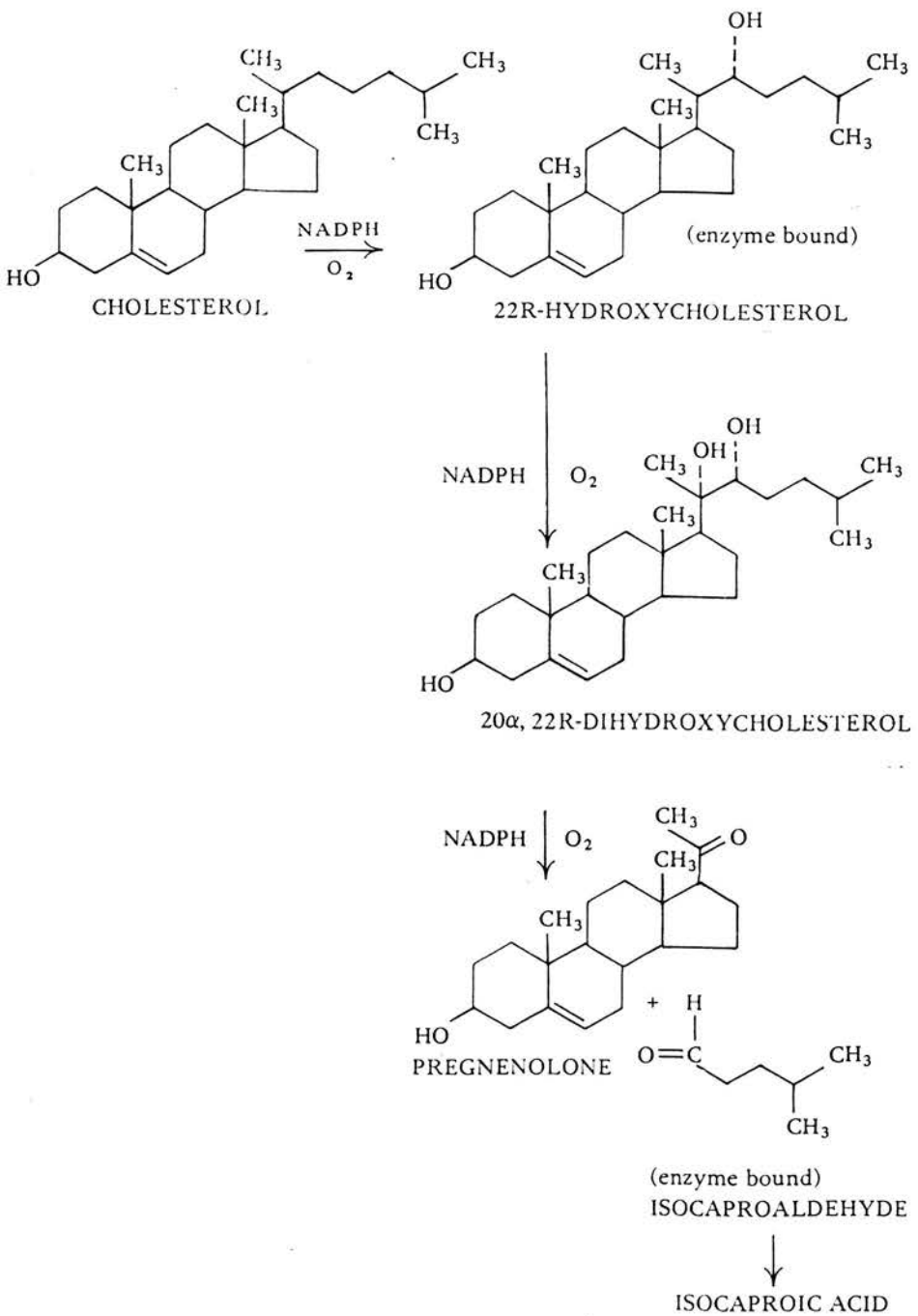


Figure 1.4. Diagram depicting the conversion of cholesterol to pregnenolone.

enzymes, determined by rates of gene transcription, stability of mRNA and translation rates is also a factor. An increase in all steroidogenic enzymes will lead to a general increase in steroid production. Each of these parameters can be regulated together or separately by steroidogenic agonists.

Five cytochrome P450 enzymes (P450scc; P450c17; P450c21; P45011β and P450aldo) and a steroid dehydrogenase enzyme (3-β-hydroxysteroid dehydrogenase (3βHSD) are involved in corticosteroid synthesis from cholesterol. The cytochrome P450 enzymes are located in the mitochondria and endoplasmic reticulum of most tissues and contain an active site, which contains an heme group, and binds the substrate. The enzymes either catalyse a single hydroxylation at a specific position or a series of consecutive hydroxylations resulting in C-C bond cleavage or aromatisation of the steroid ring (Hanukoglu *et al.*, 1992).

Once cholesterol has been transported from the cytosol to the inner mitochondrial membrane P450scc (cholesterol side-chain cleavage), catalyses the conversion of the 27-hydrocarbon cholesterol to the 21-hydrocarbon pregnenolone via three successive monooxygenations, C20 hydroxylation, then C22 hydroxylation and finally cleavage of the C20-C22 bond. Although cholesterol transfer across the inner mitochondrial membrane limits steroid flux overall, this enzymatic step is the rate-limiting step in steroid biosynthesis. This step occurs at the start of all three steroidogenic pathways. P450scc exists as a complex with adrenodoxin and adrenodoxin reductase, which act as electron transport proteins, transferring electrons from NADPH to the terminal oxidase P450scc. The enzyme has been localised to the inner mitochondrial membrane and is present in all three zones of the cortex (reviewed by Ishimura & Fujita, 1997).

3β-hydroxysteroid dehydrogenase (3βHSD) converts pregnenolone and 17α-hydroxypregnenolone to progesterone and 17α-hydroxyprogesterone respectively.

The enzyme catalyses a two step reaction, namely dehydrogenation of 3β-hydroxy-5-ene-steroid and isomerisation of 3-oxo-ene-steroid. This enzyme has been localised in the same cells as P450scc, in all three zones of the adrenal cortex, and appears to be situated in the membrane of the smooth endoplasmic reticulum (reviewed by Ishimura & Fujita, 1997).

The enzyme P450c17, (17α-hydroxylase /17, 20 lyase), catalyses two separate reactions, 17α-hydroxylation of 21-hydrocarbon steroids and cleavage of the C17-C20 bond of 21-hydrocarbon steroids. The first reaction is involved in mineralocorticoid and glucocorticoid synthesis, i.e. the formation of 17α-hydroxypregnenolone and 17α-hydroxyprogesterone. The second reaction is essential for the biosynthesis of androgens, resulting in formation of DHEA from 17α-hydroxypregnenolone, and androstenedione from 17α-hydroxyprogesterone. The latter occurs in the rat but not the human (reviewed by Hanukoglu, *et al.*, 1992). The factors determining which activity, lyase or hydroxylation, P450c17 performs remian unclear. It has been proposed that lyase activity is dependent on the availability of electrons to P450c17. Alternatively, a cAMP-dependent phosphorylation of serine and threonine residues on P450c17 has been shown to be important for lyase activities (reviewed by Miller *et al.*, 1997). The enzyme has been localised within the smooth endoplasmic reticulum in the ZF and ZR, with no presence within the ZG. Most rodent adrenals do not have this enzyme and thus corticosterone is the major glucocorticoid to be produced.

P450c21 (21-hydroxylase) catalyses the formation of the precursors of cortisol and corticosterone, i.e. deoxycortisol and deoxycorticosterone, from 17α-hydroxyprogesterone and progesterone respectively, and is expressed in all three zones of the adrenal cortex (reviewed by Ishimura & Fujita, 1997).

P45011β (11β-hydroxylase), is responsible for the formation of cortisol from deoxycortisol and corticosterone from deoxycorticosterone, and exists in a complex with adrenodoxin and adrenodoxin reductase. It is localised within the inner mitochondrial membranes of cells in all three cortical zones in the bovine adrenal, although higher concentrations are found in the ZF and ZR. In fact the bovine enzyme exists as two isoforms, one present in the ZG and the other in the ZF and ZR (White *et al.*, 1992). The ZG isoform catalyses the formation of aldosterone via 11β-hydroxylation of deoxycorticosterone, followed by 18-hydroxylation and 18-oxidation. The 18-hydroxylation and 18-oxidation steps are suppressed in the ZF and ZR thus stopping aldosterone synthesis in these zones. Within the rat adrenal this enzyme appears to be localised exclusively within the ZF and ZR. Thus the rat ZG only synthesises low amounts of corticosterone through the action of P450aldo. A distinct enzyme, encoded by a separate gene with 11β-hydroxylation, 18-hydroxylationa and 18-oxidation activities exists in the rat and human ZG which is responsible for aldosterone synthesis. P45011β can also hydroxylate androstenedione, forming 11β-hydroxylated androgens (reviewed by Ishimura & Fujita, 1997)

A single enzyme catalyses the last three steps in the synthesis of aldosterone. P450aldo (aldosterone synthase), catalyses the 11β-hydroxylation and 18-aldehyde conversion of deoxycorticosterone to aldosterone. The enzyme has similar properties to P45011β but is encoded by a different gene. CYP11B2 encodes for P450aldo and CYP11B1 encodes for P45011β. These two genes lie in tandem 50kb apart on chromosome 8 and are highly homologous (>95% nucleotide sequence) (Boon *et al.*, 1997). In sheep and pigs only CYP11B1 is found, scattered throughout the whole adrenal cortex, and it may be responsible for synthesis of both cortisol and aldosterone. In the cow however five CYP11B1 enzymes have been detected, only two cDNAs have been isolated. These encode two isozymes that differ by three amino acids (Boon *et al.*, 1997). CYP11B1

extracted from bovine adrenal cortex can synthesise aldosterone *in vitro*, however *in situ*, only the enzyme located within the ZG can synthesise aldosterone. So aldosterone producing activity is blocked in those mitochondria that are located within the ZF and ZR (Muller *et al.*, 1998). CYP11B2 has been localised to the mitochondria of rat and hamster ZG cells (reviewed by Ishimura & Fujita, 1997).

1.6. Metabolism of Aldosterone.

Steroids are rendered biologically inactive and available for excretion by five main reactions, reductions, hydroxylations, side-chain cleavage, oxidations and esterifications. In general metabolism occurs in the liver, but can also occur in the kidney and adrenal gland.

Aldosterone is metabolised in a number of ways and the key metabolites are shown in Figure 1.5. Aldosterone content of the adrenal glands is in the range of 1-2μg, while its secretion rate is 70-250 μg/day, yielding plasma levels of 5-100 pg/ml. Aldosterone metabolism occurs primarily in the liver where 85% is inactivated. The initial reaction, a reduction of the A ring and the 3-ketone group, forms tetrahydroaldosterone, which is then esterified at the 3-hydroxyl position with glucoronic acid and then secreted. In the kidney, conjugation of the 18 carbon position with glucoronic acid, without alterations of the A ring and the 3-ketone group can occur. This forms a labile product in dilute acid and therefore free aldosterone is released by hydrolysis at pH 1. Urinary measurements of aldosterone refer to this compound.

1.7. Actions of Aldosterone.

Although the clinical importance of the adrenal cortex in the regulation of electrolyte balance has long been realised, it was not until the early 1950's that the major regulatory steroid, aldosterone, was isolated and purified by Simpson *et al.*,

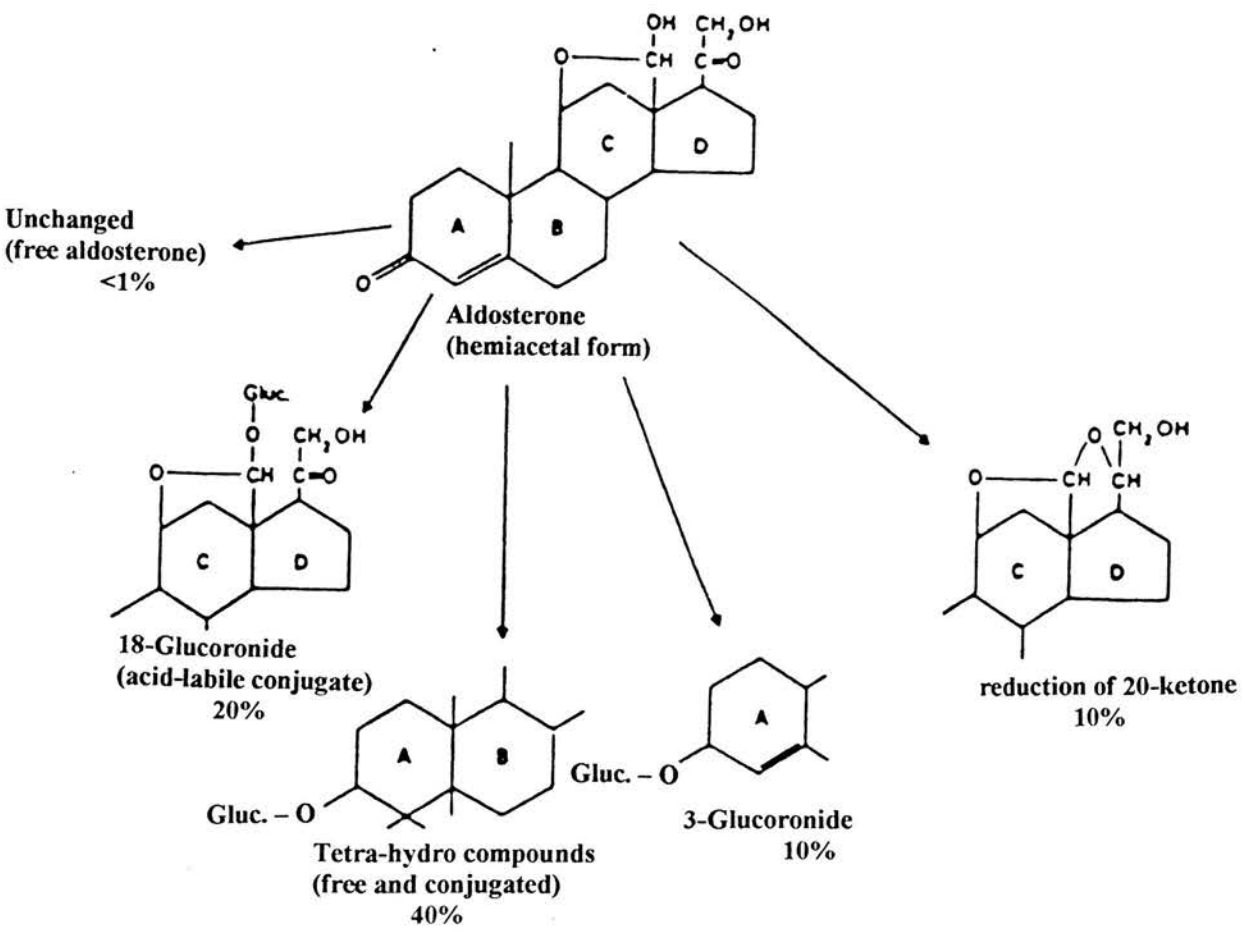


Figure 1.5. Diagram depicting the metabolism of aldosterone.

(1953), using a sensitive mineralocorticoid bioassay based on changes in urinary sodium and potassium ratios in adrenalectomised rats to characterise aldosterone.

Aldosterone is the most potent mineralocorticoid known, secreted solely by the ZG of the adrenal cortex. Other steroids with mineralocorticoid activity are 11-deoxycorticosterone and corticosterone, but although they are produced by the adrenal in much greater quantitites, they are approximately thirty-fold and three hundred-fold less potent than aldosterone, respectively. Aldosterone acts predominantly on the distal convoluting tubules and the collecting ducts of the kidney, although extra-renal sites of action include the sweat, gut and salivary glands. The half-life of aldosterone is ~twenty to thirty minutes, and its circulating concentration is low (~200-800pM). This may be explained in part by the fact that once aldosterone is synthesised it is immediately released from the adrenal gland, only a small amount is actually stored, and once in circulation it is weakly associated with plasma proteins such as corticosteroid binding globulin (~20%) and albumin (~40%), the rest being non-bound or "free".

The action of mineralocorticoids on electrolytes is essentially to conserve sodium and eliminate potassium. The effect seen is a result of the complex action of these hormones on different tissues and organs, and these vary widely with different cell types. The overall action of aldosterone depends markedly on sodium status. Increased sodium intake results in more tubular sodium available for reabsorption, thus enhancing potassium excretion. Conversely, sodium restriction diminishes both sodium reabsorption and kaliuresis. Potassium itself can stimulate aldosterone secretion, leading to eventual potassium excretion; thus aldosterone serves as an intrinsic part of the body's defence mechanism against hyperkalaemia. An additional mechanism protects against persistant mineralocorticoid excess, whereby the body 'escapes' from further sodium retention and hyperkalaemia. This is

thought to be mediated via secondary increases in other factors, such as atrial natriuretic hormone (ANH) and by renal haemodynamics.

Aldosterone binds to the mineralocorticoid receptor to trigger cellular events. The mineralocorticoid receptor is a member of the steroid/thyroid/retinoid superfamily of intracellular receptors and was cloned in 1987 (Arriza *et al.*, 1987; Evans *et al.*, 1988). These are ligand-dependent transcription factors and thus the effects of aldosterone are mediated through the regulation of the expression of specific mineralocorticoid receptor responsive genes (Evans *et al.*, 1988). In the absence of hormone the receptor resides predominantly in the cytoplasm, in a complex with heat-shock proteins (Evans *et al.*, 1988). When aldosterone binds there is a conformational change in the receptor's structure, the heat-shock proteins dissociate and the receptor migrates to the nucleus where it binds to the promoter region of target genes and regulates transcription from these genes (Fejes-Toth *et al.*, 1998).

The mineralocorticoid receptor can also be activated by cortisol and corticosterone. The glucocorticoids bind with the same high affinity as aldosterone, however *in vivo* the mineralocorticoid receptor specifically binds aldosterone in tissues such as kidney and colon (Funder *et al.*, 1988). Circulating levels of cortisol are usually 2 to 3 orders of magnitude greater than those of aldosterone, so mechanisms must exist to allow the specific activation of this receptor by aldosterone. The most important of these mechanisms involves the action of the type 2 isoform of the enzyme 11β hydroxy-steroid dehydrogenase (11βHSD2) (White *et al.*, 1997). This enzyme converts cortisol and corticosterone into inactive 11-reduced metabolites in mineralocorticoid receptor responsive tissues, such as the kidney and colon, and protects the receptor from glucocorticoids.

The electrophysiological or biochemical response to aldosterone involves an initial latent period of 30 to 60 minutes, followed by an acute phase of 1 to 3 hours, and then a chronic phase of 3 to 6 hours onwards (Verrey *et al.*, 1998). In the chronic phase a number of secondary responses occur, which after several days results in morphological changes in the distal nephron (Wade *et al.*, 1990). The latent phase reflects the time taken for the transcription and then translation of the specific gene-product or aldosterone-induced proteins. The identity of these putative aldosterone-induced proteins has proven elusive, although there are strong candidates (Verrey *et al.*, 1998). The acute phase reflects the physiologic response to aldosterone-induced proteins before other factors, which may include gene expression, make a significant contribution.

Steroid hormones may also act through alternative nongenomic pathways. Several groups have now reported rapid effects, within minutes, of aldosterone on intracellular pH, calcium dependant Na^+/H^+ exchange and cardiovascular function *in vivo* (Christ *et al.*, 1995; Gekle *et al.*, 1996; Wehlin *et al.*, 1998).

The importance of aldosterone as a regulator of electrolyte and fluid balance in both normal and diseased states has led investigators to study its mechanism of action, as a firm understanding of the biochemical mechanisms of aldosterone action could facilitate development of new methods and new drugs in the treatment of disorders of mineralocorticoid secretion. Increased quantities of aldosterone and its metabolites are present in the urine of patients with a number of diseases, including primary and secondary hypertension, congestive heart failure, cirrhosis of the liver and nephrosis. Mineralocorticoid deficiency causes sodium loss, hyperkalaemia and acidosis. One disease state linked with both mineralocorticoid and glucocorticoid deficiency is Addison's disease.

1.8.Physiological Control of Aldosterone Secretion.

Numerous factors affect aldosterone secretion, acting directly or indirectly. To discuss them all is outwith the scope of this thesis, and so only the major regulatory factors, and those relevant to this thesis will be discussed.

1.8.1. The Renin-Angiotensin System.

Since the discovery of renin almost 100 years ago, there have been remarkable advances in the understanding of the renin-angiotensin system (RAS). The RAS is thought to be the major physiological regulatory component of aldosterone secretion, and parallel changes in plasma renin activity (PRA) and aldosterone secretion under a variety of conditions confirm this (Davis *et al.*, 1975; Fraser *et al.*, 1979).

1.8.1.1. Formation of Angiotensin II.

Renin is a proteolytic enzyme stored in intracellular granules as both the inactive prorenin and active renin in the juxtaglomerular cells of the afferent arteriole of the kidney. Renin secretion is controlled by neurogenic, haemodynamic and humoral mechanisms (reviewed in Valotton, 1987). Once stimulated it is released exocytotically and diffuses into the circulation. After release, renin acts on a globulin plasma protein, angiotensinogen, cleaving it at the leu10-leu11 bond to form a decapeptide, angiotensin I (AI) which has relatively low biological activity. Angiotensin converting enzyme (ACE), an endopeptidase found in many tissues but predominantly in the lung, cleaves AI at a leucine and a histidine residue to form an octapeptide, AII. Angiotensinogen can also be directly converted to AII by the action of serine protease tonin, which is found in the venous affluent of the submaxillary gland. AII has a very short half - life and is rapidly broken down by aminopeptidases in various tissues to produce inactive peptide fragments. Aminopeptidase A removes the N-terminal aspartate residue from AII to form the heptapeptide angiotensin III (AIII) (Reviewed by Espiner & Nichols, 1993).

1.8.1.2. Mechansims of Action of Angiotensin II

AII has a multiplicity of actions, initiating a complex series of events which work together to restore plasma volume, blood pressure and sodium concentration. It is a potent pressor agent through direct vasoconstrictor actions, stimulation of the sympathoadrenal system both centrally and peripherally and via inhibition of vagal control of heart rate. AII also controls fluid and electrolyte balance, stimulates drinking and salt appetite and directly affects the renal handling of sodium. However its ability to regulate aldosterone secretion is the key pathway through which the RAS controls fluid and electrolyte balance.

AII exerts its action on target organs by interacting with receptors in the plasma membrane (Catt *et al.*, 1993). AII receptors are coupled to cellular second messengers such as G-proteins, ion channels and membrane enzymes. The receptors show a high binding affinity and rapid association and dissociation of ligands. The receptors can undergo changes in concentration which has a knock on effect on the actions of circulating AII. Sodium intake can alter the concentration of AII receptors in the ZG and the vascular tissue. Sodium depletion increases AII receptors, while sodium loading decreases them in the ZG, but has the opposite effect in the vasculature. Thus the adrenal responsiveness to AII is increased by sodium depletion, while the vascular response is decreased. The observation that both sodium depletion and high potassium diets, known stimuli of aldosterone production, increase levels of the adrenal AT_1 receptor may be explained by the up-regulation of AT_1 gene expression by aldosterone (Inagami *et al.*, 1995).

AII binds to one of two major receptor types, AT_1 and AT_2 receptors. They belong to the class of seven transmembrane receptors coupled to G-proteins (Timmermans *et al.*, 1993). The AT_1 receptor has two isoforms AT_{1A} and AT_{1B}, exhibiting minor

differences in amino acid sequences. In contrast the AT_1 and AT_2 receptors exhibit only 32-34% homology (Csikos *et al.*, 1998).

Most of the biological actions of AII are mediated through the AT_1 receptor, probably via the AT_{1A} receptor, apart from in the adrenal where control of aldosterone secretion appears to be mediated through the AT_{1B} receptor (Gigante *et al.*, 1997). The AT_2 receptor does not influence aldosterone secretion.

AII stimulation of aldosterone secretion requires calcium in the extracellular fluid and can be blocked by calcium channel blockers. AII stimulates calcium influx and efflux and rapidly elevates the intracellular calcium concentration by mobilising calcium from intracellular and extracellular sources. This flux of calcium is the result of AII binding to the receptor coupled by G-protein and then activation of phospholipase C. Phospholipase C breaks down polyphosphoinosotide to diacylglycerol and inositol 1,4,5-triphosphate. The inositol 1,4,5-triphosphate mobilises calcium from intracellular endoplasmic reticulum stores and the diacylglycerol activates protein kinase C. Stimulation of calcium influx by AII may be through voltage-gated or receptor-mediated calcium channels (Franco-Saenz *et al.*, 1989). The initial rise in intracellular calcium activates calcium calmodulin dependent protein kinases and is responsible for the early steroidogenic response to AII. Sustained release of aldosterone requires influx of calcium via T-type voltage dependent channels as well as increased activity of diacylglycerol sensitive protein kinase C. In addition AII activates phospholipase A_2 resulting in the release of arachidonate and its metabolic products, prostaglandin and leukotriene.

Aldosterone secretion is enhanced within minutes of AII stimulation (reviewed by Lumbers, 1999). In acute doses, AII acts on the early stages of the aldosterone biosynthetic pathway, i.e. prior to formation of pregnenolone. This is true for all known stimulants of aldosterone secretion. After chronic exposure, AII affects

later stages of the biosynthetic pathway, i.e. on aldosterone synthase, thus AII causes a rise in plasma aldosterone levels *in vivo*, which can be sustained for hours (Blair-West *et al.*, 1970). In preparations from K^+ deficient, sodium-loaded or mineralocorticoid treated rats, that is animals who are secreting higher than normal rates of aldosterone, AII does not stimulate aldosterone secretion, instead plasma deoxycorticosterone levels increase (Muller *et al.*, 1998). Aldosterone synthase mRNA disappears from the ZG of potassium deplete, sodium loaded or mineralocorticoid treated rats and markedly increases in sodium restricted rats or potassium loaded rats (Muller *et al.*, 1978; Shibata *et al.*, 1991; Tremblay *et al.*, 1992). The increase in aldosterone synthase mRNA can be totally or partially blocked by treatment with an ACE inhibitor, indicating that induction of aldosterone synthase depends on either systemic or locally formed AII (Shibata *et al.*, 1991). In the rat potassium loading induces transcription and expression of aldosterone synthase in the ZG but has no effect on P45011β in the ZF. Thus potassium status determines the extent to which circulating AII modulates aldosterone secretion, through interactions at the level of expression of aldosterone synthase (reviewed by Lumbers *et al.*, 1999).

AII is converted to AIII and it is believed that AIII has similar actions to AII, although the plasma concentrations of AIII are much lower. The combined stimulatory effects of AII and AIII on aldosterone secretion results in inhibition of renin release from the kidney and as a consequence aldosterone secretion from the ZG. This is an important negative feedback mechanism involved in the regulation of aldosterone, sodium homeostasis and blood pressure.

1.8.2. Potassium.

Potassium is another physiological regulator of aldosterone secretion. Low plasma levels of potassium lead to a decrease in aldosterone secretion, and vice versa, via a direct action on the ZG (Blair-West *et al.*, 1970; Tait *et al.*, 1972; Braley *et al.*,

1986). In acute doses, potassium stimulates the early step in the aldosterone biosynthetic pathway, i.e. prior to formation of pregnenolone, chronically potassium stimulates the conversion of deoxycorticosterone to aldosterone, and increases the width of the ZG. In the rat potassium loading induces transcription and expression of aldosterone synthase in the ZG, but has no effect on P45011β in the ZF, thus a major site at which potassium controls aldosterone secretion is through the control of aldosterone synthase (reviewed by Lumbers *et al.*, 1999).

A number of experiments have shown a direct action of potassium on the ZG cell and aldosterone production, both *in vivo* and *in vitro* (Boyd, *et al.*, 1971; 1973). Aldosterone production is exquisitely sensitive to small changes in potassium concentration. The state of potassium balance can also alter the response of aldosterone to AII. Linde *et al.*, (1981) reported that aldosterone secretion in humans on a high potassium diet (300 mequiv./day) was more sensitive to AII infusions than in subjects on an 80 or 10 mequiv. potassium diet. In another study potassium depleted rats failed to respond to sodium depletion, with an increase in aldosterone production or an increase in the width of the ZG (Boyd *et al.*, 1973). The plasma renin levels were not lowered, indicating a loss of sensitivity to AII as a result of potassium depletion. Ikubo & Ichikawa (1997) produced several strains of 'knock-out' mice that were deficient in a specific gene within the RAS and found unequivocal evidence that potassium is an effective regulator of aldosterone secretion during extracellular fluid volume depletion in the absence of the RAS.

At first it was thought that the translocation of extracellular potassium into adrenal ZG cells by way of the Na^+/K^+ pump was a prerequisite for the aldosterone stimulating effect of potassium (Boyd *et al.*, 1973). However, unlike other aldosterone secretagogues, potassium causes depolarisation of the plasma membrane, activating voltage-dependent calcium channels in the plasma membrane as the initial transduction step (Kojima *et al.*, 1985). This increase in calcium

influx, due to increasing concentrations of potassium, results in a rise in cytosolic free calcium that is well correlated with aldosterone production (Capponi *et al.*, 1984; Braley *et al.*, 1986). Measurements of Ca^{++} with Quin 2 demonstrated that the increase in calcium influx was rapid and sustained for the duration of the stimulation. Nifedipine completely inhibited the aldosterone response to potassium, indicating a role for extracellular calcium and a voltage-dependent calcium channel. $^{45}Ca^{++}$ influx and efflux studies confirmed that the calcium is of extracellular origin and is not mobilised from an intracellular source (Fakunding *et al.*, 1979; Williams *et al.*, 1981). However cytosolic calcium responses to potassium can be inhibited by nitrendipine, dantrolene and TMB 8, suggesting that release of cytosolic bound calcium may be involved in the response. However these agents may have other uncharacterised effects on calcium metabolism besides blockade of membrane bound calcium release (Bradley *et al.*, 1986).

The cAMP response to potassium is controversial. Kojima *et al.*, (1985), reported a small increase in cAMP in calf adrenocortical cells and suggested that potassium mediated aldosterone secretion may involve a dual mechanism requiring both cytosolic free calcium and cAMP participation. However Ganguly *et al.*, (1985), found no increase in cAMP levels in calf adrenal ZG cells. The weight of evidence suggests that potassium affects cAMP generation, an effect prevalent at higher concentrations of potassium, although it is not clear whether this is due to changes in cytosolic calcium levels, or a direct effect of potassium itself. No increase in radiolabelled IP_3 or tritiated inositol incorporation into phospholipids is observed in the presence of increased concentrations of potassium (Whitley *et al.*, 1984). However, a small transient decrease in radiolabelled PIP_2 has been reported and an increase in the mass of IP_3 lasting for only ten seconds was observed when ZG cells were incubated with 8.7mM of potassium (Kojima *et al.*, 1985; Underwood *et al.*, 1987).

1.8.3. Sodium.

Restriction of sodium intake is one of the most potent stimuli for aldosterone secretion in man and in animals (Davis, 1967). Sodium depletion not only increases aldosterone secretion but also the width of the ZG, while sodium loading decreases both of these factors. The mechanisms by which alterations in sodium balance leads to changes in aldosterone secretion remain unclear, mainly due to the diverse physiological effects initiated by altered sodium status. Plasma sodium levels have little or no direct effect on aldosterone production. The rise in aldosterone secretion in sodium deficient animals depends on the rise of AII levels, yet sodium deficiency alters the sensitivity of the adrenal to AII. This may be due to effects of sodium deficiency on local or paracrine factors, e.g. the adrenal RAS.

Sodium deficiency activates the RAS causing a rise in plasma AII levels, stimulating aldosterone production. The RAS clearly plays an important role in the aldosterone response to changes in sodium, it seems clear however that this is not the only mechanism involved. The effects of AII on the adrenal are enhanced by sodium deficiency. Sodium loading of a previously salt deplete animal results in a more rapid reduction in plasma aldosterone levels than in plasma AII levels (Boon *et al.*, 1997). *In vitro* studies have shown that sodium directly inhibits the adrenal response to AII, but this effect is probably due to osmotic effects as aldosterone does not increase when renin levels are elevated in water deplete animals (Schneider *et al.*, 1984; 1985). Boon *et al.*, (1997), found that a major 4 kb transcript of P45011β in the sheep ZG and also in the ZF increased with potassium loading and rose further with acute sodium depletion, but did not increase further with chronic sodium depletion. They suggested that the rise in aldosterone levels in moderately sodium deficient sheep was due to an increased supply of deoxycorticosterone and the increased aldosterone synthase levels were due to stimulation by AII. They postulated that in severe sodium depletion there was a switch in the aldosterone biosynthetic pathway allowing dissociation from AII stimulation. They also

suggested that other factors such as the adrenal RAS system could be involved. In nephrectomised animals, aldosterone output is still enhanced during sodium restriction, which may be due to the involvement of a pituitary derived substance, not ACTH, which is secreted during sodium depletion and stimulates aldosterone secretion (McCaa *et al.*, 1974; Pratt *et al.*, 1981).

With the use of continuous superfusion and perfusion techniques, both *in vivo* and *in vitro*, Baniukiewicz *et al.*, (1968), demonstrated that alterations in sodium balance can influence aldosterone production at different stages of the biosynthetic pathway, in sheep and in rats; namely at the conversion of corticosterone to aldosterone and at a step preceding the formation of corticosterone. Adrenal tissue of sodium deplete dogs was shown to convert more unlabelled and radioactively labelled progesterone and corticosterone to aldosterone than adrenal tissue of sodium replete dogs (Davis *et al*, 1968). Marusic & Mulrow (1967), found an increased conversion of corticosterone to aldosterone and 18-hydroxycorticosterone, but a normal conversion of deoxycorticosterone to corticosterone by capsular adrenal mitochondria of rats that had been kept on a sodium deficient diet for four days.

1.8.4. ACTH

ACTH is the major regulator of glucocorticoid production, but also has a transient stimulatory effect on aldosterone, stimulating aldosterone secretion both *in vivo* and *in vitro*, acting at the early stage of the biosynthetic pathway (Davis *et al.*, 1975; Fraser *et al.*, 1975). However in the rat chronic treatment with ACTH markedly reduces aldosterone production both *in vivo* and *in vitro* (Muller *et al.*, 1970; 1978). In fact after continuous infusion of ACTH, aldosterone levels return to normal within 1 to 3 days, while corticosterone levels remain elevated.

The release of ACTH during acute animal experiments can mask the effect of other systems on aldosterone secretion. Following hypophysectomy in rats the ZG does not atrophy, unlike the inner zones (Deane, 1962). Deane *et al.*, (1948) demonstrated that a low sodium diet increases the width of the ZG in hypophysectomised rats. Chronic hypophysectomy in a number of species does not markedly influence sodium balance, however acute injections of ACTH leads to a marked increase in aldosterone secretion and chronic ACTH administration does not (Mulrow *et al.*, 1966). ACTH plasma levels do not increase following sodium depletion, but ACTH is necessary in a permissive way in maintaining the integrity of the cell and in the early steps of the biosynthesis of aldosterone, i.e. cholesterol to pregnenolone. Loss of ACTH in sodium depleted rats results in a significant decrease in aldosterone production (Boyd *et al.*, 1972). Following sodium depletion and potassium loading there is increased sensitivity of aldosterone secretion to ACTH (Yamakodo *et al.*, 1983).

A number of suggestions have been made as to the possible mechanisms of the effects of ACTH in aldosterone biosynthesis, falling into two distinct categories. Firstly, those involving the direct effects of ACTH on the adrenal ZG cells, and secondly, those that are secondary to the effects of ACTH on other ZG modulators. Direct effects of ACTH on the ZG include functional and morphological transformations of ZG cells into either intermediate or fasciculata-like cells (Hornsby *et al.*, 1985; Muller *et al.*, 1978; McDougall *et al.*, 1980), and an intra-adrenal inhibition of the late steps of aldosterone biosynthesis by inappropriately increased levels of aldosterone biosynthesis (Muller, 1970; Aguilera *et al.*, 1981). The indirect effects that have been suggested include increases in the volume of the extracellular fluid compartment due to increased levels of steroids with weak mineralocorticoid activity, i.e. deoxycorticosterone, 18-OH-deoxycorticosterone and corticosterone, leading to a reduction in the activity of the RAS (Aguilera *et al.*, 1978), a negative feedback inhibition of an as yet unidentified aldosterone

stimulating factor from the pituitary (Legros & Lehoux, 1983) and a fall in plasma potassium concentrations (Aguilera *et al.*, 1981). However from studies investigating the chronic inhibitory actions of ACTH it would appear that a direct action of ACTH on the ZG is responsible for the reduced aldosterone biosynthesis (Abayasekara *et al.*, 1989).

Receptors for ACTH are expressed throughout the ZG and the ZF (Mountjoy *et al.*, 1992). The receptor is coupled to adenylate cyclase by a stimulatory G-protein, which binds GTP and stimulates adenylate cyclase and cAMP production respectively (Kojima *et al.*, 1985b). The adrenal steroidogenic response to ACTH is dependent on the presence of extracellular calcium. Calcium is also closely associated with the activation of the adenylate cyclase system. In the absence of extracellular calcium, cAMP and aldosterone secretion are inhibited, and ACTH has a greater effect on cAMP production when extracellular calcium is elevated (Fakunding *et al.*, 1979; Shima *et al.*, 1979). The former effect was later attributed to the requirement of Ca^{2+} for the binding of ACTH to its receptor (Cheitlin *et al.*, 1985).

1.9. Other Regulators of Aldosterone Secretion.

1.9.1. Innervation of the Adrenal Cortex.

Direct innervation of endocrine cells, in the mammalian adrenal, was illustrated by both Unsicker *et al.*, (1971) and Garcia-Alvarez *et al.*, (1970), utilising a catecholamine fluorescence technique, demonstrating innervation of the basal lamina of cortical cells by autonomic axons, terminating close to the plasma membrane. Two sources of innervation have been proposed for the rat adrenal. Firstly, from the adrenal medulla, the adrenal cortex may have originally been a target organ for adrenomedullary postganglionic nerves, and so during evolution as the two tissues became more closely associated, the postganglionic fibres terminated entirely within the adrenal gland, forming the chromaffin tissue and

innervating the cortical tissue (Hinson, 1990). Secondly, nerves which appear to have cell bodies outwith the adrenal gland, entering the gland along blood vessels (Holzwarth *et al.*, 1987). These adrenal nerves entering the gland contain both pre- and postgangloinic fibres, and the postganglionic fibres supply the adrenal cortex (Kesse *et al.*, 1988; Carlsson *et al.*, 1990). Sympathetic preganglionic cholinergic fibers reach the adrenal gland by the splanchnic nerves which traverse the cortex and synapse on chromaffin cells, where they regulate catecholamine synthesis and release (Neville *et al.*, 1969). Stimulation of these nerves may also increase the levels of certain neuropeptides such as vasoactive intestinal peptide and neuropeptide Y, within the medulla and in a paracrine manner, possibly within the cortex (Bloom *et al.*, 1988). Adrenocortical innervation includes extrinsic sympathetic catecholaminergic nerves and intrinsic nerves, predominantly vasoactive intestinal peptidergic nerves. Neuropeptide Y fibers also appear associated with blood vessels (Kong *et al.*, 1989).

Numerous neuropeptides are found within the adrenal gland, and transmitter systems have been identified in nerves supplying the adrenal cortex. Immunohistochemistry has demonstrated the presence of several amines and peptides within the adrenal cortex nerves. Catecholamines, tyrosine hydroxylase and dopamine-β-hydroxylase, which are localised in nerve fibers within the adrenal capsule and ZG (Oomori *et al.*, 1991; Vizi *et al.*, 1993). Also neuropeptides such as vasoactive intestinal peptide, neuropeptide Y, galanin and calcitonin gene related peptide, also localised within the adrenal capsule and ZG (Toth *et al.*, 1995). In sheep corticotrophin releasing hormone has also been identified in nerve fibers within the adrenal cortex (Rundle *et al.*, 1988).

The distribution of the various types of nerve fibers occurs in the outer part of the cortex, mainly within the capsule and the ZG, and so two distinct possibilities exist, firstly, blood flow regulates steroidogenesis, that is, the major regulatory role of the

neurotransmitters on steroidogenesis could be indirectly mediated by an action on the vasculature system, and/or, the neurotransmitters released could also have a direct effect on adrenocortical cells and steroidogenesis. Also, the splanchnic nerve plays an important role in regulation of adrenocortical nerve activity. As has already been discussed, splanchnic nerve stimulation releases many important neuropeptides into the adrenal venous effluent. However at present it is unclear how much of this is due to cortical innervation as opposed to the adrenal medulla. The neuropeptides secreted are not only present within the nerve fibres innervating the cortex but also within the chromaffin cells of the adrenal medulla.

1.9.2. Cortical-Medullary Interactions.

1.9.2.1. Co-Existence of Cortical and Medullary Cells.

More than twenty years ago, Palcois & Lafarga (1975), reported the existence of islets of chromaffin cells in the zona glomerulosa of adult rats, although they put this observation down to an error in adrenal organogenesis rather than a functionally significant observation. However, strong evidence is now available from morphological and immunohistochemical studies on the adrenal gland, in various species, as to the co-localisation of cortical cells and medullary cells.

In the rat adrenal, rays of medullary tissue have been reported to extend across the outer zones of the cortex, up to the capsular zone, some of these rays actually follow the connective fibres of the large adrenal vein, and even after cell isolation the interaction between cell types was still seen (Gallo-Payet *et al.*, 1987). Chromaffin cells, containing organelles identical to adrenocortical mitochondria and smooth endoplasmic reticulum, were detected in adrenals of perfusion fixed rats, and it is thought that these cortico-chromaffin hybrid cells may have the capacity to carry out steroid biosynthetic reactions (Bornstein *et al.*, 1991). Immunohistochemical studies in the pig adrenal gland using chromagranin A revealed the existence of chromaffin cells projecting from the medulla across all

three cortical zones (Bornstein *et al.*, 1991). Also evident were small islets and single neuroendocrine cells in the ZF and ZR. Immunostaining for 17α-hydroxylase stained immunoreactive cells in the medulla. In the bovine adrenal, cortical cells could also be detected in the medulla especially around the blood vessels. In the human adrenal gland Bornstein *et al.*, (1994) identified the occurrence of chromaffin cells in all three zones of the adrenal cortex. Ultrastructural analysis demonstrated that the cortical and chromaffin cells were in close apposition, not separated by fibrous tissue, and so sufficient contact between the cells exists to allow widespread paracrine interactions, and in some rare instances chromaffin cells appeared to be releasing their secretory products by exocytosis near adrenocortical cells. The close colocalisation of the two cell types forms the basis for many interactions between the two endocrine systems.

1.9.2.2. Paracrine Control of Adrenocortical Function by the Adrenal Medulla.

The medulla is innervated by preganglionic, cholinergic and sympathetic neurones carried in the splanchnic nerves. Stimulation of the splanchnic nerve results in the release of splanchnic nerve products within the medulla which may affect the adrenal cortex. It has now been shown that numerous adrenomedullary secretory products, including catecholamines, 5-HT, ANP, somatostatin, substance P, vasoactive intestinal peptide (VIP), neuropeptide Y (NPY), adrenomedullin and pituitary adenylate-cyclase activating peptide (PACAP), are involved in the regulation of adrenocortical steroidogenesis, by either stimulating or inhibiting adrenocortical function.

1.9.2.2.1. Catecholamines and 5-HT.

Bornstein and co-workers (1990) demonstrated that in isolated perfused pig adrenals with a preserved nerve supply, splanchnic nerve activation stimulated the release of corticosteroids, specifically cortisol, aldosterone and androstenedione. This stimulatory effect may have been mediated by chromaffin cells in a paracrine

manner, as perfusion of the adrenals with catecholamines also provoked a significant release of corticosteroids. In addition to this acute effect, catecholamines have a long-term effect on corticosteroid release in the rat from isolated adrenocortical cells, involving transcriptional regulation of steroid enzymes (Erhart-Bornstein *et al.*, 1998). In the frog adrenal gland, catecholamines had the reverse effect, inhibiting steroid secretion. It has also been suggested that catecholamines regulate the synthesis of aldosterone in the ZG (De Lean *et al.*, 1984). Although adrenaline has apparently no effect on intact adrenal glands *in vitro.*, it did stimulate steroidogenesis in regenerating medullaectomised rat adrenal glands. In addition medullary products were also found to affect steroidogenesis in the inner zone cells of the adrenal cortex, for example, catecholamines and ATP both stimulate the production of cortisol (Walker *et al.*, 1988).

5-HT has been localised within the adrenal medulla, specifically the chromaffin cells, of various species, namely mouse, rat and frog. It is now well known that 5-HT stimulates corticosteroid secretion from the adrenal cortex in various animal models, and thus this is another example of a neurotransmitter present within the adrenal medulla which could influence adrenocortical activity (Reviewed by Nussdorfer, 1996).

1.9.2.2.2. Medullary Neuropeptides.

Medullary chromaffin cells produce, store and secrete not only catecholamines but also a whole range of neuropeptides, which either act to inhibit or stimulate steroidogenesis, depending on the circumstances involved. In the main these peptides stimulate adrenocortical function in different species, thus influencing adrenal steroid production. ANP, a regulator of aldosterone secretion, somatostatin, dynorphin, substance P, NPY and the enkephalins are all thought to have an inhibitory role on steroidogenesis. However, in certain circumstances, substance P and the enkephalins, act to stimulate steroidogenesis (reviewed by

Erhart-Bornstein *et al.*, 1998). ANP and somatostatin, preferentially act on the release of mineralocorticoids within the ZG.

1.9.2.2.3. Corticosteroids.

Corticosteroids are known to have effects on adrenaline synthesis and release in the medulla. Steroid hormones secreted from the adrenal cortex exert selective trophic actions on the medulla promoting growth, maturation and maintenance of chromaffin cells, as well as modulating the activity of the constitutive enzyme, phenylethanolamine N-methyltransferase (PNMT), most characteristic of the medulla.

The medulla and cortex would appear to interact widely with complex regulatory circuits. Under basal conditions, stimulatory influences appear to be dominant. When physiological conditions differ, different secretory products come into play and it remains to be elucidated as to how the different adrenomedullary secretory products are involved in the control of adrenocortical functions (reviewed by Erhart-Bornstein *et al.*, 1998).

1.9.2.3. Gap Junctions.

Gap junctions allow water soluble molecules to pass directly from the cytoplasm of one cell to the cytoplasm of the other, propagating the intracellular signal from stimulated to unstimulated cells, thus coupling cells both electrically and metabolically. They have been found to occur within all zones of the mammalian adrenal cortex, providing an effective cellular communication system (Murray & Pharrams, 1997). With the use of an interactive laser cytometer the existence of gap junctions in bovine adrenocortical cells in culture has also been visualised. These functionally active gap junctions mediate paracrine signals between chromaffin and cortical cells. Furthermore, gap junctions are induced after

stimulation with ACTH, which suggests a possible role in response to the hormone (Murray & Pharrams, 1997).

1.9.3. The Intra-Adrenal Renin-Angiotensin System.

For years the RAS was viewed as a classical circulating endocrine system. There now exists local tissue or organ specific RAS that acts in a paracrine or autocrine fashion. The physiological role of such systems remains unknown, although they may serve as a mechanism for limiting the actions of AII to a specific organ or tissue.

A renin-like enzyme was first identified in rabbit adrenal by Ryan and co-workers (1967), which reacted with a renin substrate to form AI. Many studies have now established renin-like activity in a wide number of tissues, including the heart, adrenal glands, liver, vasculature, brain and testes (Phillips *et al.*, 1993). In the adrenal cortex, renin has been found in rabbits, dogs, cattle, rats and humans (Wang *et al.*, 1992), and mRNA for renin has been detected in rats (Dzau *et al.*, 1987) and mice (Field *et al.*, 1984). In the rat adrenal gland each participating component in the formation of AII has been identified (Mulrow, 1998). However, tissue systems remain controversial due to the notion that much of the renin present in such tissues arises from uptake of kidney renin, rather than by *in situ* synthesis. But in sodium depleted or potassium loaded rats, biosynthesis and secretion of adrenal renin, localised mainly in the ZG was increased (Doi *et al.*, 1995). Under these physiological conditions, the increase in renin activity was demonstrated to be linked to elevated levels of renin mRNA indicating that adrenal renin is synthesised locally rather than being taken up from the extensive vascular system supplying the gland (Wang *et al.*, 1992).

Direct evidence for AII formation within the adrenal gland has arisen from various studies. AII was secreted from superfused rat and human adrenal glands, and

stimulation of both AI and AII, by rat tissue, was stimulated by increased potassium concentrations (Kifor *et al.*, 1991; Sarzani *et al.*, 1992). Superfusion of rat ZG cells by potassium produced similar results. Chronic stimulation by low dietary sodium, increased both the output of AII per cell and the number of cells involved in AII secretion (Chiou *et al.*, 1995).

Although the physiological role for the intra-adrenal RAS has not been established, many observations have shown that the concentration of renin in the ZG parallels that of aldosterone. In cultured rat ZG cells, ACTH, AII and potassium stimulated both aldosterone secretion and renin activity. The increase in renin activity correlated with increased levels of renin mRNA, indicating an increase in the transcriptional rate of the renin gene or an increase in the stability of the mRNA (Wang *et al.*, 1992). The activity of the intra-adrenal RAS may affect the density of adrenal AII receptors. Gigante *et al.*, (1997), found that losartan administration to bilaterally nephrectomised salt restricted rats caused the expected rise in adrenal renin mRNA and activity and a fall in aldosterone synthase. Also AT_{1B} receptor mRNA levels fell, while AT_{1A} mRNA levels were unchanged and AT_2 mRNA increased. It has been suggested that the intra-adrenal RAS is involved in the control of P450scc and P45011β gene expression in response to sodium restriction or potassium loading. P45011β is dependent solely on the presence of AII, whereas P450scc is modulated by several factors including AII, emphasising the key part played by this peptide in the control of steroidogenesis (Reviewed by Erhart-Bornstein *et al.*, 1998).

1.9.4. Dopamine.

Since the initial observation that the dopamine agonist bromocriptine, partially blocked frusemide stimulated aldosterone synthesis (Edwards *et al.*, 1975), there has been controversy surrounding the physiological importance of dopamine in the regulation of corticosteroidogenesis.

A possible role for dopamine in regulating aldosterone secretion came largely from pharmacological *in vivo* experiments using the dopamine antagonist metoclopramide, which enhanced aldosterone secretion but not PRA, ACTH or plasma potassium, indicating that under normal circumstances aldosterone secretion is under tonic inhibition by dopamine (Carey *et al.*, 1979). The dopaminergic mechanism appears to be independent of the nervous system as adrenal catecholamine content is not depleted by splanchnic nerve section or central inhibitors of catecholamine biosynthesis (McCarty *et al.*, 1984). It has been suggested that dopamine exerts a tonic inhibitory influence on aldosterone secretion (Carey *et al.*, 1984). Other workers have suggested that inhibition of aldosterone synthesis by dopamine was an extra-adrenal effect caused by increased renal clearance of AII (Connell *et al.*, 1987). Further studies, using a rat adrenal cell perfusion system, demonstrated that the steroid enhancing effects of metoclopramide, could be blocked by dopamine but not AII (Carey *et al.*, 1979; Edwards *et al.*, 1980a). Aguilera & Catt (1984), then demonstrated that during sodium repletion the sensitivity of the aldosterone response to AII was enhanced in the presence of metoclopramide, suggesting that the antagonist reversed the inhibitory effect of dopamine. A similar effect was seen in humans, although infusions of dopamine in humans, together with AII, prevented the increase in sensitivity to AII normally seen in low sodium states (Edwards *et al.*, 1975; Aguilera & Catt, 1984). Conversely, other groups have demonstrated no stimulatory effect of metoclopramide on aldosterone secretion both *in vivo* and *in vitro*, also no inhibitory effect of dopamine on aldosterone secretion (Campbell *et al.*, 1981; Hampton & Ganguly, 1986). Also the 5-HTergic properties of metoclopramide itself must be considered, making interpretation of these studies difficult. Metoclopramide is a substituted benzamide prokinetic drug, and in high concentrations blocks 5-HT_3 receptors, but at least part of its prokinetic action can be ascribed to its agonist activity at 5-HT_4 receptors, which are coupled to

adenylate cyclase (Dumuis *et al.*, 1988). *In vitro* studies incubating isolated rat and bovine ZG cells with dopamine are not conclusive either. In these studies, dopamine had no effect or a moderate inhibitory effect on basal aldosterone secretion, and in cells stimulated with AII or ACTH, aldosterone secretion was not affected, a slight inhibition was sometimes seen at high concentrations ($>10^{-5}$M)(Edwards *et al.*, 1980a, McKenna *et al.*, 1979; Aguilera & Catt, 1984). In contrast, infusions of AII plus dopamine in sodium deplete humans impeded the increased sensitivity to AII normally observed. This regulatory role of dopamine during altered sodium status has been supported by other studies which have demonstrated a decrease in urinary dopamine during sodium depletion and conversely an increase during sodium loading (Alexander *et al.*, 1974; Carey *et al.*, 1981). Porter *et al.*, (1992), demonstrated that dopamine infusions in the isolated *in situ* perfused rat adrenal for ten minutes resulted in a transient dose-related decrease in aldosterone secretion (~50% of the basal at 10^{-6}M dopamine). Simultaneous administration of haloperidol, a dopamine antagonist, reversed the effect of dopamine, without affecting basal aldosterone release. If the gland was electrically field stimulated the aldosterone secretion observed was decreased, and this effect was also abolished by haloperidol. The investigators suggested that the local release of dopamine may be involved in the inhibitory effects, thus providing evidence for a physiological control of the ZG by dopamine.

The effects of dopamine on the adrenal are thought to be mediated by specific dopamine receptors located on the ZG, classified as DA_1 and DA_2 subtypes. The mechanism of dopaminergic action is not entirely understood, though some groups have suggested that the DA_1 receptor stimulates adenylate cyclase activity and aldosterone production, whilst the DA_2 receptor inhibits adenylate cyclase and aldosterone (Missale *et al.*, 1986). With the discovery of these two receptor subtypes, the seemingly contradictory results produced from the various groups, on dopamine control of aldosterone secretion, may be reconciled. The two receptor

subtypes are present within the adrenal cortex, and mediate opposing effects, and the net effect of dopamine on steroidogenesis may depend on which response predominates in the particular preparation (Gallo-Payet *et al.*, 1990). Thus inhibition of aldosterone secretion in the perfused rat adrenal may be attributed to interaction of dopamine with the DA_2 receptor. Also the failure of much higher concentrations of dopamine to affect ZG cells has been related to its ability to stimulate both subtypes.

The dopamine involved in the control of aldosterone secretion could potentially be derived from the circulation or the adrenal itself as several groups have shown the presence of dopamine containing granules within the adrenal gland, in many species. Further support for the local effect are studies demonstrating the stimulatory effects of metoclopramide infusion on aldosterone secretion after blockade of the autonomic nervous system (Wilson *et al.*, 1983). *In situ* formation of dopamine may occur, as the presence of the enzyme L-AAAD has been demonstrated within the adrenal gland, and so circulating L-DOPA may be converted to dopamine within the gland, and act as a local paracrine mechanism for modulating aldosterone secretion.

1.9.5. 5-HT.

The stimulatory effects of the indoleamine serotonin (5-hydroxytryptamine, 5-HT) on steroid secretion by the adrenal cortex were originally described by Rosenkrantz (1959). From 1962 onwards, a series of studies demonstrated that pineal gland extracts were able to stimulate aldosterone secretion *in vitro* (Farrell *et al.*, 1962). The active substance was identified as 5-HT in 1967 (Jouan, 1967).

Since these early reports many groups have shown the aldosterone stimulatory properties of 5-HT on the ZG, both *in vivo* and *in vitro*, in many species. No effect has been reported on the ZF (Muller *et al.*, 1970; Haning *et al.*, 1970).

In the rat early studies, proposed that the 5-HT induced aldosterone secretion was mediated by a 5-HT_2 receptor positively coupled to cAMP (Matsuoko *et al.*, 1985; Williams *et al.*, 1984). It is now well documented that activation of 5-HT_2 receptors causes stimulation of phospholipase C, with no effect on cAMP formation (Conn *et al.*, 1986). Also 5-HT has no effect on inositol phosphate formation within ZG cells of the rat (Rocco *et al.*, 1990). So it is clear that the effect of 5-HT on aldosterone secretion cannot be mediated through activation of 5-HT_2 receptors. In frog and human adrenocortical cells the receptor site present within the ZG is the 5-HT_4 receptor (Lefebvre *et al.*, 1992; Contesse *et al.*, 1994). Contesse *et al.*, (1999) characterised the rat receptor as the 5-HT_7 receptor, present within the ZG.

5-HT induces a significant stimulation of cAMP production in frog, rat and human adrenal preparations (Lefebvre *et al.*, 1992; Matsuoko *et al.*, 1985). Microfluorimetric studies have shown that in frog and human adrenocortical cells 5-HT_4 agonists cause elevation of the cytosolic calcium concentration, due to calcium influx through T-type membrane channels, which is secondary to the cAMP activation (Hamel *et al.*, 1996; Contesse *et al.*, 1996). Similarly it has been shown that 5-HT stimulates $^{45}Ca^{++}$ uptake by rat ZG cells (Davies *et al.*,1991). No change in calcium efflux was observed from an intracellular source in isolated ZG cells loaded with $^{45}Ca^{++}$ stimulated with 5-HT, suggesting that 5-HT may act in a similar fashion to ACTH (Williams, *et al.*, 1981).

In addition to its direct effect 5-HT can also stimulate aldosterone release by increasing adrenal blood flow (Hinson *et al.*, 1989). 5-HT may also modulate interleukin-6 and tumour necrosis factor secretion from the adrenal ZG (Ritchie *et al.*, 1996). As these two cytokines can both modulate corticosteroid production, they may in part mediate the corticotropic effects of 5-HT (Tominaga *et al.*, 1991). In amphibians, 5-HT acts directly on adrenocortical cells to stimulate corticosterone

and aldosterone secretion in a dose-dependent manner (Delarue *et al.*, 1988). In humans, *in vitro* studies have shown that 5-HT stimulates corticosterone secretion by adrenal cells derived from patients with Cushing's disease and aldosterone secretion by adrenal cells from aldosterone-producing adenomas (Shenker *et al.*, 1985). Also it has been shown that 5-HT triggers cortisol secretion from perifused human adrenal explants (Lefebvre *et al.*, 1992). The use of a perifusion system model, which provides detailed information on the kinetics of the response of adrenocortical cells to corticotropic factors has revealed that sustained administration of 5-HT causes a biphasic stimulation of cortisol secretion. Initially there is a rapid and transient increase followed by a rapid decline of cortisol release (Lefebvre *et al.*, 1992), indicating that 5-HT, like other regulatory signals, induces a desensitisation process.

Intravenous injection of 5-HT and/or oral administration of tryptophan and 5-HTP to normal volunteers causes a significant increase in plasma corticosteroid levels and ACTH (Mantero *et* al., 1982; Modlinger *et al.*, 1979; Shenker *et al.*, 1985). But do 5-HT and its precursors exert their effects on the adrenal ZG *in vivo* at the hypothalamic, pituitary, renal or directly at the ZG level. *In vivo*, 5-HT stimulates ACTH secretion and activates the RAS. Studies have demonstrated that 5-HT causes stimulation of corticotrophin releasing factor from rat hypothalamic tissue (Fuller & Clemens, 1981; Holmes *et al.*, 1982). However isolated cell preparations, which have no central involvement, produce aldosterone when stimulated directly with 5-HT. In dexamethasone treated volunteers, after oral 5-HT_4 agonist treatment, there is a significant rise in aldosterone levels, with no alterations in plasma renin levels, potassium concentration, ACTH and cortisol (Lefebvre *et al.*, 1993; 1995). This would seem to indicate that the aldosterone stimulatory activity of 5-HT *in vivo*, cannot be accounted for by activation of the RAS, elevation of potassium or stimulation of pituitary corticotrophs.

The occurrence of 5-HT within the adrenal gland has been demonstrated by immunohistochemical and biochemical approaches. 5-HTergic fibers have been detected in the mouse adrenal cortex (Fernandez-Vivero *et al.*, 1993), and the occurrence of 5-HT has been immunocytochemically and biochemically demonstrated in the adrenal medulla of mice and rats (Verhofstad & Jonsson, 1983; Fernandez-Viveros *et al.*, 1993). In the rat and frog adrenal gland, 5-HT like immunoreactivity has been visualised in phenylethanolamine-N-methyl-transferase-positive cells, indicating that only epinephrine producing cells contain 5-HT (Brownfield *et al.*, 1985; Delarue *et al.*, 1988). In fact, electron microscope studies revealed that 5-HT is sequestered in secretory vesicles located at the periphery of the chromaffin cells. Thus 5-HT is likely to be released during splanchnic nerve stimulation together with catecholamines. In the rat 5-HT also occurs in mast cells, located in the wall of arterioles at the point they enter the connective capsule of the adrenal gland, and throughout the medulla and cortex (Hinson *et al.*, 1989). In humans chromaffin cells do not contain 5-HT, intra- and perivascular mast-like cells which are present throughout the adrenal gland are the only store of 5-HT (Lefebvre *et al.*, 1992). HPLC analysis of mouse, frog, rat and human adrenal gland extracts, combined with electrochemical detection, has revealed the presence of 5-HT and 5-HIAA. The occurrence of 5-HIAA within adrenal tissue most likely reflects local metabolism of 5-HT after release of the indoleamine from serotoninergic cells. Within the human adrenal cortex mast cells secrete 5-HT which is then metabolised by a type A monoamine oxidase located in intracortical chromaffin cells (Lefebvre *et al.*, 1996).

Verhofstad & Jonsson, 1983, reported that 5-HT in the rat adrenal gland was formed by decarboxylation of 5-HTP. In the frog interrenal gland, substantial amounts of 5-HT and 5-HIAA are synthesised from [^{3}H]-tryptophan, suggesting that tryptophan hydroxylase activity is expressed within frog chromaffin cells (Delarue *et al.*, 1992). Active uptake of exogenous 5-HT by chromaffin cells has

been demonstrated in frog, cattle and rat adrenal glands (Delarue *et al.*, 1992; Verhofstad & Jonsson, 1983).

1.10. 5-HT, biosynthesis, metabolism and receptor sites.

This next section will serve as an overall review of 5-HT, briefly describing the biochemistry, metabolism, transport and action, mainly in the adrenal cortex, of the indoleamine 5-HT. At the end of this section the main aims of this thesis will be discussed.

1.11. Biosynthesis and Metabolism of 5-HT.

Apart from 5-HT neurones in the brain, spinal cord and in the gut (enteric plexus), the major site of 5-HT synthesis in man is in the enterochromaffin cells of the gut (Erspamer & Testini, 1959). These cells have also been described as amine precursor uptake and decarboxylation, or APUD, cells (Pearce, 1968). Platelets, which contain a high concentration of 5-HT, do not synthesise 5-HT, but in fact accumulate 5-HT from the plasma by a high affinity uptake system. Thus under normal circumstances plasma contains a relatively low concentration of free 5-HT. 5-HT is synthesised from dietary L-tryptophan in two enzymatic steps, via the intermediate compound 5-hydroxytryptophan (5-HTP) (Figure 1.6). In almost all tissues that contain large amounts of 5-HT, apart from platelets, the enzyme tryptophan hydroxylase has been detected, so the enzyme has been located in the enterochromaffin cells, the pineal gland, mouse mast cells and the CNS (Lovenberg *et al.*, 1965; Deguci, 1977; Legay *et al.*, 1983). Inside the cell the enzyme activity can be located in the cytosol or associated with subcellular organelles (Lovenberg *et al.*, 1967). Centrally 5-HT is synthesised from L-tryptophan entering the brain through the blood-brain barrier and is not dependent upon peripheral 5-HT synthesis. Circulating 5-HT does not enter the brain tissue through the blood-brain barrier, except perhaps locally in areas deficient in this barrier such as the area postrema (Grahame-Smith,1987). The formation of 5-HT, both centrally and

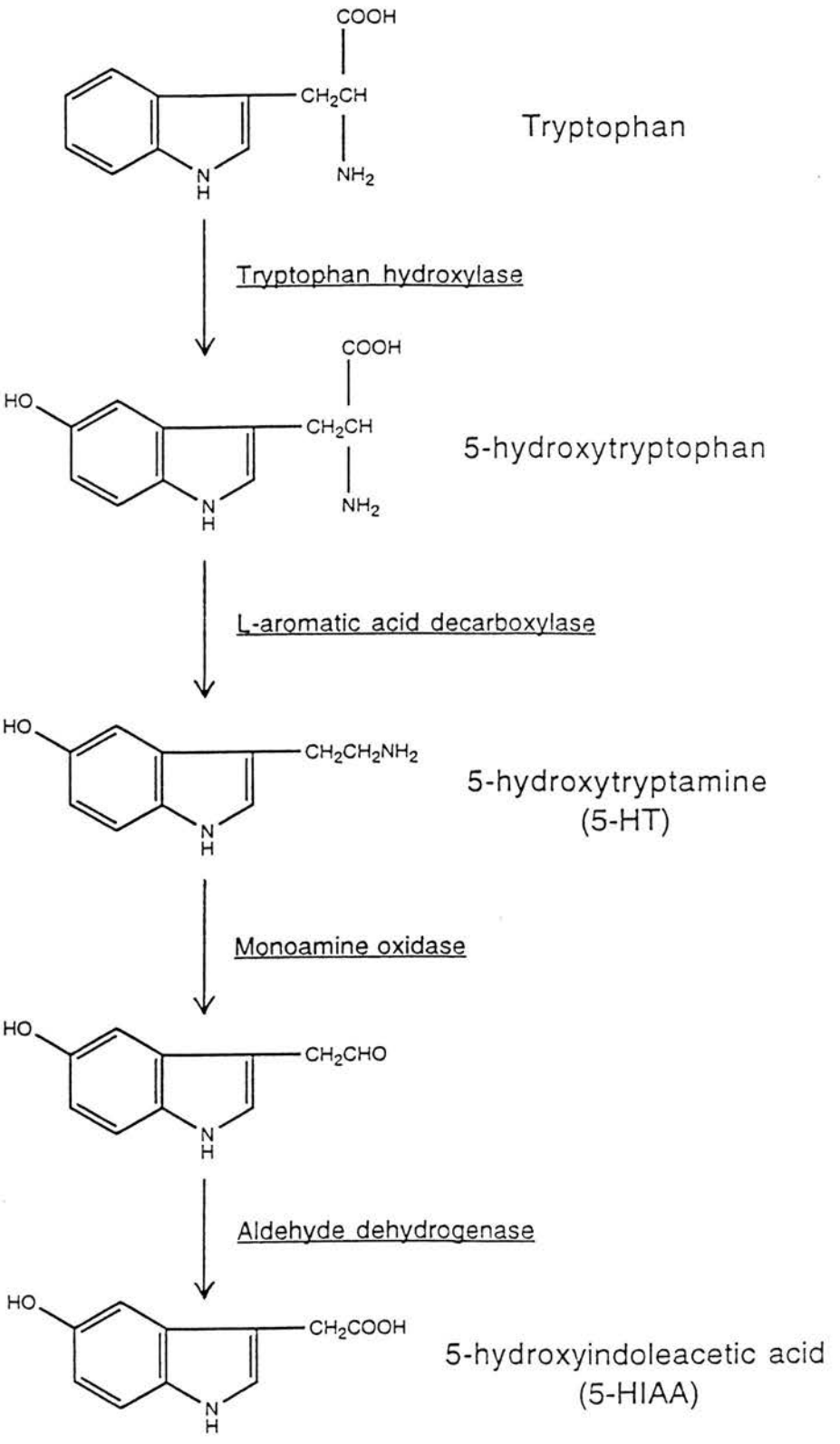


Figure 1.6. Diagram depicting the biosynthesis and metabolism of 5-HT.

peripherally, is not a major metabolic pathway for tryptophan, as only 1-2% of dietary tryptophan is converted to 5-HT. Tryptophan circulates in the blood in both free and albumin bound forms. Since the enzyme converting tryptophan to 5-HTP, tryptophan hydroxylase, is not saturated with substrate *in vivo* (Km ~ $3x10^{-5}M$) under normal circumstances, and since 5-HTP is rapidly decarboxylated into 5-HT, the rate-limiting step in 5-HT biosynthesis is the 5-hydroxylation of tryptophan by tryptophan hydroxylase (Lovenberg *et al.*, 1967). The hydroxylation of tryptophan into 5-HTP requires the presence of a co-factor, a tetrahydrobiopterin, and it has been suggested that the concentration of the oxidised form of this co-factor (dihydrobiopterin) may also play a regulatory role in 5-HT synthesis. However, an increase in tetrahydrobiopterin concentration *in vitro* did not lead to an increase in 5-HT synthesis. At concentrations of $2x10^{-4}M$ tryptophan, the enzyme is inhibited. In addition the pharmacological agent p-chlorophenylalanine (PCPA), inhibits the enzyme and thus provides a specific tool for inhibiting 5-HT synthesis which can be used to investigate the effects of 5-HT depletion (Reviewed by Smith, D.G., 1988).

The second enzymatic step in the formation of 5-HT is the conversion of 5-HTP into 5-HT catalysed by the enzyme L-aromatic amino acid decarboxylase (L-AAAD; Figure 1.6). The presence of L-AAAD was first reported in mammalian kidney extracts (Holtz *et al.*, 1938) and is located in a number of tissues including liver, kidney, brain and adrenal gland. L-AAAD has a Km of $8x10^{-6}M$, which is quite low and thus this enzymatic step in the formation of 5-HT is not rate-limiting. The co-factor involved is pyridoxal phosphate, which if present in excess of $6x10^{-7}M$ inhibits L-AAAD activity (Bouchard & Roberge, 1979). L-AAAD is also involved in the production of catecholamines, such as dopamine, trace amines such as tyramine from tyrosine, 2-phenylethylamine from phenylalanine and tryptamine from tryptophan (Juorio & Boulton, 1982; Dyck *et al.*, 1983). In addition, L-AAAD may have as yet unknown functions in non-monoamine neurones in which

L-AAAD has been localised (Jaeger *et al.*,1986; Kitahama *et al.*, 1988; Eaton *et al.*, 1993) (See Chapter Five).

5-HT undergoes several metabolic transformations (Figure 1.7). Oxidation of 5-HT, by monoamine oxidase type A, forms 5-hydroxyindoleacetaldehyde, an unstable intermediate. This enzyme is located on the outer mitochondrial surface and requires NAD^+ as co-factor (Tipton *et al.*, 1976). Further oxidation by aldehyde dehydrogenase produces 5-hydroxyindole acetic acid (5-HIAA), large quantities of which are excreted in urine (up to 50 μmol/day; Udenfriend, 1956). 5-HT is metabolised in this way in the liver and lungs. The liver also contains a detoxificating enzyme, glucoronyltransferase, which conjugates glucoronic acid to 5-HT as well as other compounds including lipids. 5-HT-O-glucoronide is a major metabolite in isolated rat liver perfusate and if monoamine oxidase activity is inhibited 5-HT is readily converted to this product (Tyce *et al.*, 1968). In addition, if the oxidative pathway, which forms 5HIAA, is inhibited, say after ingestion of alcohol, an alternative reductive pathway occurs, catalysed by aldehyde reductase, forming 5-hydroxytryptophol (Davis *et al.*, 1967). This enzyme requires NADP as co-factor, has a high affinity for its substrate and is a minor metabolic pathway in mammals. Another metabolic pathway is conversion into N-acetyl 5-HT, by the enzyme 5-HT-N-acetyltransferase, which is located in the cell cytosol and requires acetyl co-enzyme A (Paul *et al.*, 1974). N-acetyl 5-HT is then converted into 5-methoxy-N-acetyltryptamine (Melatonin), by hydroxyindole-O-methyltransferase which utilises S-adenosylmethionine as the methyl donor. Formation of melatonin occurs primarily in the pineal gland, and is of particular interest since there is a marked rhythm in the levels of melatonin in the blood and also in the activity of the enzyme N-acetyltransferase, which is responsible for the synthesis of N-acetyl 5-HT from 5-HT. The peak of N-acetyltransferase activity occurs during the hours of darkness, and coincides with peak levels of 5-HT in the pineal gland. Melatonin is responsible for blanching the skin of amphibians and suppressing gonadal activity in

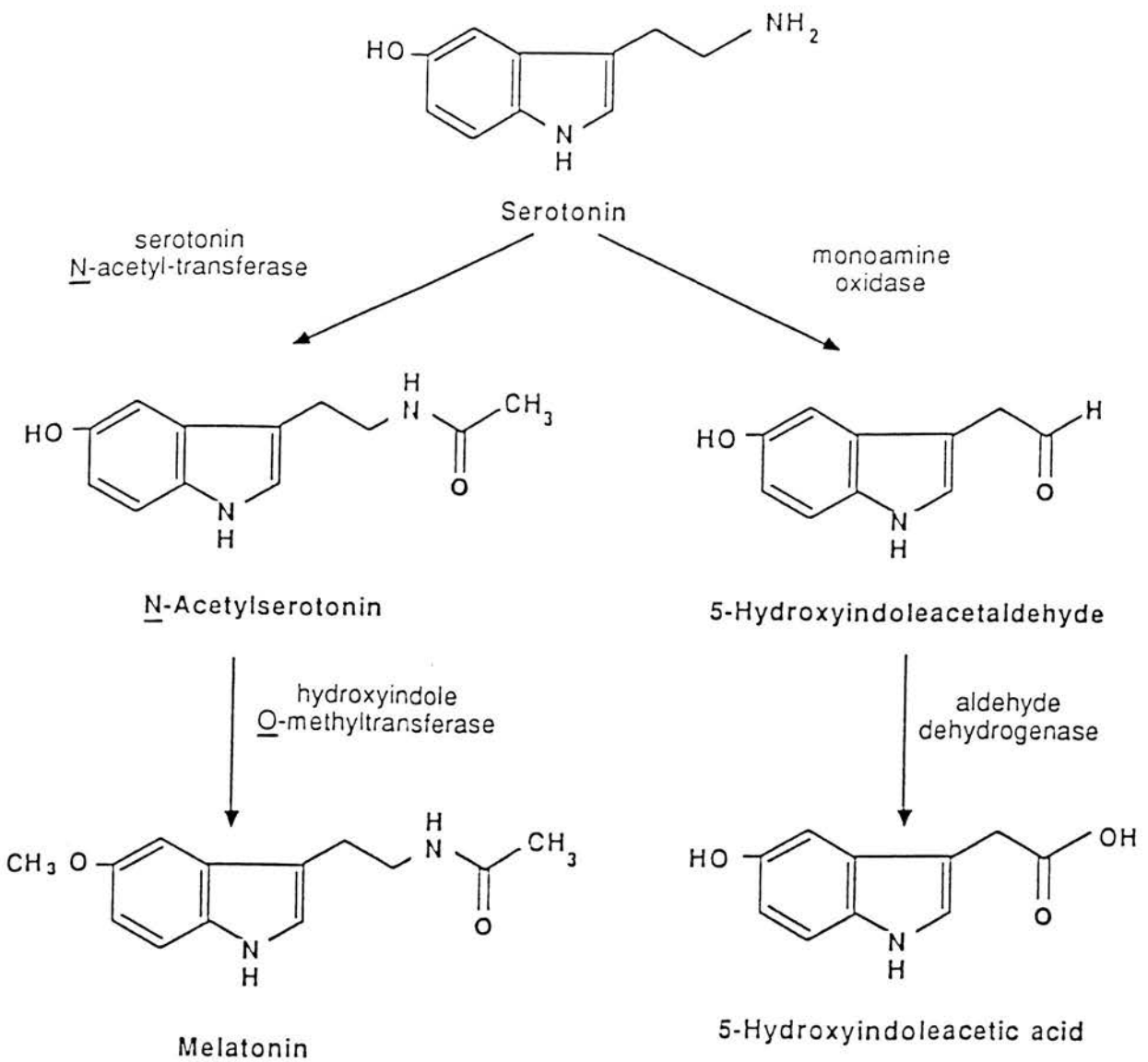


Figure 1.7. Diagram depicting the metabolism of 5-HT.

mammals. The enzyme hydroxy indole-O-methyl transferase can also act on 5-HT to form 5-methoxy 5-HT, and this has been detected in the hypothalamus of the rat (Green *et al.*, 1973). 5-HT can be conjugated to a sulphate moiety by 5-HT sulfotransferase, an enzyme which has been located in liver, lung, kidney and the CNS. Sulphonated 5-HT, 5-HT-O-sulphonate, has little biological activity, while removal of the sulphate group by arylsulphatase can regenerate free 5-HT (Kishimoto *et al.*, 1961). This metabolite accounts for a small proportion of 5-HT metabolites, and its concentration does not increase markedly after monoamine oxidase inhibition. Another minor metabolic pathway, occurring in the lung and CNS, is the N-methylation of 5-HT which requires the enzyme S-adenosylmethionine.

1.12. The Transport and Storage of 5-HT.

1.12.1 Transport of 5-HT.

The levels of biogenic amines in the synaptic cleft, such as 5-HT, are regulated by the action of plasma membrane biogenic amine transporters. These carrier proteins couple the uptake of sodium, chloride and potassium ions to the reuptake of neurotransmitters released into the synaptic cleft, thus removing extracellular transmitter, and regulating the levels of transmitter acting on the receptor. This uptake process is the first of two steps required for neurotransmitter recycling. Once the neurotransmitter is in the cytoplasm, a second transport process, requiring a hydrogen ion gradient, sequesters transmitter within synaptic vesicles, before the transmitter is again released via exocytosis. Transporters are located on the plasma membrane of serotonergic presynaptic terminals, on plasma membranes of platelets, pulmonary endothelium and placental brush border epithelia, where they participate in systemic 5-HT homeostasis (Rudnick, 1977; Balkovetz *et al.*, 1989).

The major storage site for 5-HT in the plasma is the platelet, stored within the dense granules complexed with di- and tri-nucleotides and heavy metal cations

(Pletscher, 1968). After stimulation of the gut, 5-HT is released from the enterochromaffin cells, into the bloodstream where it is available for metabolism or uptake by platelets, a process which can either be active or passive. Active uptake is energy dependent, rapid and saturable, requiring external sodium and chloride ions (Rudnick & Nelson, 1978), and internal potassium ions. Passive uptake, or diffusion, is not dependent on cellular energy, and is the dominant process at high external concentrations of 5-HT (Born & Bricknell, 1959). Once internalised, 5-HT is transported into the dense granules for storage. Once inside the dense granules 5-HT is sequestered with large aggregates of ADP, ATP and divalent metal ions.

Of the available data on the 5-HT transporter molecule most has originated from studies performed on platelet plasma membrane vesicles, as platelets are considered to accumulate and store 5-HT in a manner similar to that seen in 5-HT-ergic nerve terminals (Rudnick, 1977). Oubain does not inhibit the transport of 5-HT within platelet plasma membranes, suggesting that 5-HT transport is not directly coupled to the Na^+/K^+ ATPase, however uptake can be inhibited by tricyclic antidepressants and by ionophores such as gramicidin which catalyse transmembrane exchange of sodium and potassium dissipating the transmembrane ionic gradients (Rudnick, 1977).

A model for the transport of 5-HT is depicted in Figure 1.8. In the event of transport of 5-HT into the neurone, 5-HT, sodium and chloride ions bind to the plasma membrane transporter on the extracellular side, and this complex is then translocated to the cytoplasmic surface where it dissociates. At this point potassium ions bind to the plasma membrane transporter enabling reorientation of the protein so that the binding site once more faces the extracellular membrane surface (Graham & Langer, 1990). Inhibitors of this transporter include cocaine, imipramine, desmethylimipramine, amitriptyline, citalopram, fluoxetine and

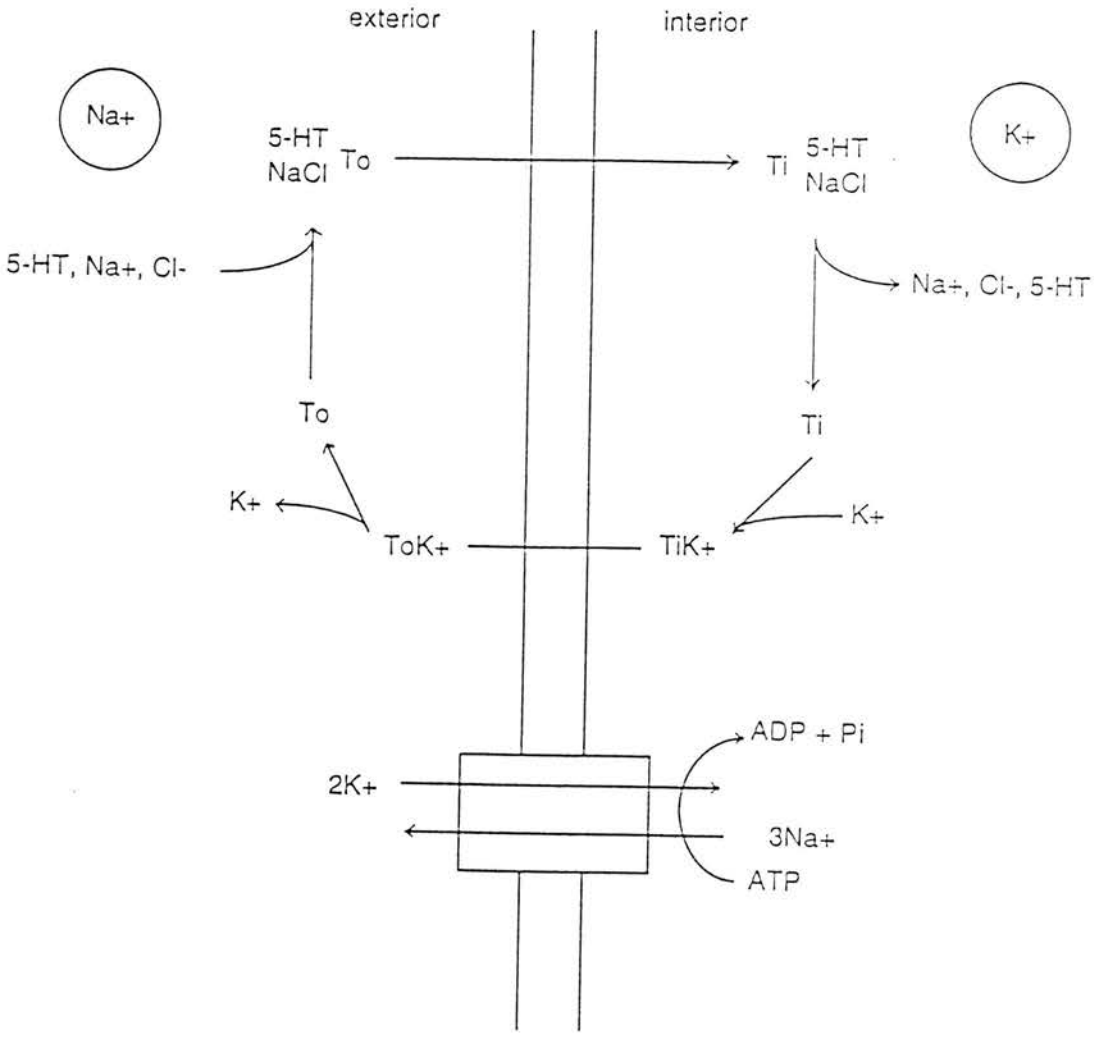


Figure 1.8. Model for the translocation cycle of the 5-HT transporter. In the first step of uptake, 5-HT, sodium ions and chloride ions bind to the transporter (To) on the exterior plasma membrane, upon which they are translocated across the membrane. After dissociation of the complex at the cytoplasmic side, the binding of potassium to the transporter (Ti), serves to reorientate the 5-HT binding site to the extracellular membrane surface (adapted from Graham & Langer, 1990).

paroxetine. Once inside the cell the uptake of 5-HT into storage vesicles, by the vesicular transporter, occurs via two sequential processes. Initially there is a vacuolar ATP-driven hydrogen ion pump which acidifies the organelle interior generating an electrochemical gradient across the vesicular membrane for hydrogen ions, the subsequent coupling of internal hydrogen ions with the vesicular transporter provides the energy for transport of 5-HT into the storage vesicle in exchange for hydrogen ion extrusion. For every protonated amine molecule taken into the vesicle, two H^+ ions are released into the cytoplasm. This uptake is blocked by reserpine and tetrabenezine. 5-HT accumulation within the storage vesicle is dependent upon a transmembrane pH difference and a transmembrane electrical potential difference (Rudnick & Clark, 1993). Synaptic vesicles, adrenal chromaffin granules, mast cells, basophilic secretory granules and platelet dense granules all contain this transport system.

1.12.1.1. Cloning of the 5-HT Transporter.

A) Plasma Membrane Transporter.

Up until the early 1990's, the absence of cloned noradrenaline transporters and 5-HT transporters cDNA's restricted various areas of study on the transporter molecule, including, gene expression, transporter structure and function, transporter regulation and biophysical properties of the transporters. In 1991 Pacholczyk *et al.*, utilised an expression cloning approach in which cDNAs were screened for their ability to confer noradrenaline transporter function in non-neuronal cells. Intracellular accumulation of the surrogate noradrenaline transporter substrate ^{125}I-labelled metaiodobenzylguanedine (MIBG) served as an autoradiographic marker for COS cells expressing the human noradrenaline transporter cDNA. The potential noradrenaline transporters identified in this study were then tested for specificity by incubating the transfected COS cells with MIBG in the presence and absence of desipramine, a noradrenaline transporter selective antidepressant. From this, a single cDNA was isolated, and, in 1991, Blakely *et al.*,

confirmed the synthesis of a catecholamine-selective transporter which was inhibited by cocaine, antidepressants and amphetamines.

At about the same time Guastella *et al.*(1990) reported the cloning of the first isoform of a Na^+ and Cl^- coupled GABA transporter, which had 46% amino acid sequence homology with the isolated noradrenaline transporter. Since the GABA and the noradrenaline transporter share sequence homology, but have markedly different pharmacological profiles, Blakely *et al.*, reasoned that transporters with similar drug sensitivities to that of the noradrenaline transporters would also exhibit ~50% sequence homology with the cloned noradrenaline transporter and thus could be isolated by homology based techniques. They were particularly interested in the 5-HT transporter, and, in 1991, Blakely *et al.* reported the cloning and expression of a 5-HT transporter within the rat brain. Northern blots and *in situ* hybridisation techniques were then used to determine whether any of these homologues might represent a 5-HT transporter. One clone hybridised selectively to a 3.7kb midbrain and brainstem RNA that exhibited localised synthesis in the serotonergic raphe complex. Isolation of a more complete midbrain cDNA, using the original isolate as a probe and looking at its function in transfected HeLa cells, confirmed the expression of a 5-HT selective transporter inhibited by selective 5-HT transporter antagonists, including paroxetine, citalopram and fluoxetine. 5-HT uptake into transfected cells was blocked by low concentrations of amphetamines and cocaine (Blakely *et al.*, 1991).

Hoffman *et al.*(1991) then described a 5-HT transporter from basophilic leukaemia cells, and since these initial studies cDNA's encoding functional 5-HT transporters have been cloned from mouse brain (Chang *et al.* 1994) and human placenta (Ramamoorthy *et al.*, 1993). The cDNA's encode proteins that are predicted to have twelve hydrophobic spanning regions. This topology is shared with other transporters, including the Na^+/glucose transporter. Among the neurotransmitter

transporters there is a great deal of sequence homology. In fact the close relationship between the GABA and noradrenaline transporters suggests that they may have all evolved from a single precursor transporter. Amino acid sequences among the neurotransmitter transporters are most similar within the hydrophobic membrane spanning regions. All of these transporters exhibit glycosylation sites on the extracellular loop between transmembrane domains three and four. Additionally, all exhibit potential phosphorylation sites on the intracellular portions of the molecule, suggesting a potential for post-transational regulation. The potential for subtypes of 5-HT transporters does exist, but the sequence identity of brain and basophilic leukaemia cDNA's indicates that peripheral and CNS 5-HT transporters are encoded by a single gene, a hypothesis supported by the identification of a single 5-HT transporter loci in human and mouse, which lies on the long arm of chromosome 17 (Gregor *et al.*, 1993; Ramamoorthy *et al.*, 1993). Also 5-HT transporters encoded by human platelet and brain RNA's appear to be identical (Lesch *et al.*, 1993).

Rat and human 5-HT transporters are predicted to be encoded by subunits of 630 amino acids with >90% cross-species identity. 5-HT transporters exhibit a topology of twelve transmembrane domains with a large extracellular loop between transmembrane domains three and four, which contains sites for n-glycosylation. NH_2 and COOH termini are predicted to lie in the cytoplasm (Figure 1.9).

B) Vesicular Transporter.

Schuldiner *et al.* (1990) isolated the transporter from detergent solubilised bovine chromaffin granule membranes using the inhibitor reserpine. The purified preparation consisted of an 80kDa glycoprotein. Transfection of CHO fibroblasts, normally sensitive to the neurotoxin MPP^+, with cDNA from PC-12 cells produced MPP^+ resistant transfectants which concentrated dopamine into vesicular structures. When the PC-12 cDNA responsible for this property was isolated and

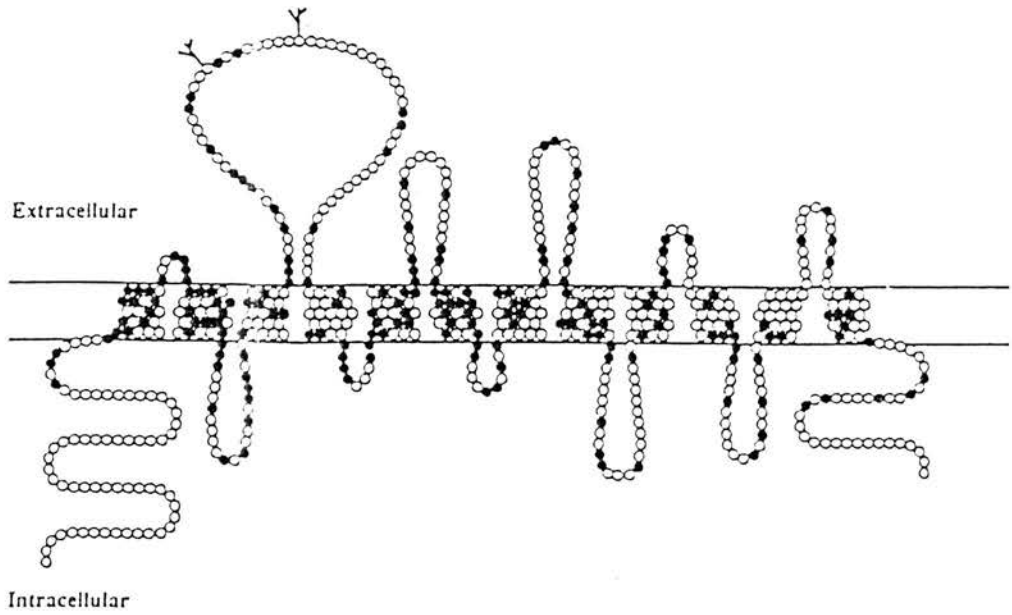


Figure 1.9. Schematic representation of the 5-HT transporter, containing 12 transmembrane domains, cytoplasmic amino and carboxy-termini, proposed N-linked glycosylation sites and residues conserved in dopamine and noradrenaline transporters (•).

sequenced, it was found to predict peptide sequences highly homologous to those of peptides derived from the purified bovine chromaffin granule amine transporter (Edwards, 1992). Hoffman *et al.*(1991), transfected CV-1 cells with cDNA prepared from RBL cell mRNA. These cells take up 5-HT using the plasma membrane transporter and store it in secretory granules containing a vesicular transporter. Some cDNA clones from this study conferred increased 5-HT uptake in CV-1 cells, and were found to encode a vesicular transporter. The sequence of this transporter is highly homologous to the transporter cloned from PC-12 cells, and almost identical to a transporter cloned from rat brain (Edwards, 1992).

1.12.1.2. Transporter Families.

At least five distinct families of transporters exist, the divisions based on differences in ion dependence, topology and sequence homology. Firstly, the sodium and chloride ion dependent transporter family which can be divided into two subfamilies; a monoamine subfamily and an amino acid-like subfamily. The monoamine subfamily consists of the dopamine, noradrenaline and 5-HT transporter molecules. These transporter molecules are all encoded by a closely related family of cloned cDNA's. and are all inhibited by cocaine and share structural and mechanistic properties. The amino acid-like subfamily consists of the GABA, glycine, betaine, taurine and proline transporters. This family is rapidly expanding and also contains a number of 'orphan clones'. The other transporter families are the glutamate transporters, the Na^+-dependent glucose transporter, the H^+-dependent vesicular monoamine transporters and a family of other vesicular transporters, with unidentified transport functions.

1.12.1.3.Distribution and Regulation of the 5-HT Transporter.

Although this transporter is closely related to the dopamine and noradrenaline transporters, substantial sequence divergence in regions between putative transmembrane domains as well as in the predicted cytoplasmic NH_2 and COOH

termini have been observed. This aspect was utilised by Blakely *et al.* (1994), to prepare 5-HT transporter antibodies to enable visualisation of the transporter *in situ*. 5-HT transporters were found to be distributed in a manner consistent with the known localisation of 5-HT uptake, 5-HT transporter antagonist binding and 5-HT immunoreactivity. Specifically, within rat and human brain, 5-HT transporters were visualised in the dorsal raphe complex, the substantia nigra and throughout the forebrain.

The mechanisms involved in the regulation of 5-HT transporters are unclear. Launay *et al.*, (1992) described the effects of histamine in elevating rates of 5-HT transport in platelets. This effect may be mediated through increases in cGMP. Activation of protein kinase C has also been implicated in platelet 5-HT transporter down regulation. *In vitro* studies in human and rat, have established that the 5-HT transporter is influenced by cAMP-dependent pathways, by changes in intracellular calcium, treatment with calmodulin inhibitors, and by activation of PKC and cGMP (Quian *et al.*, 1995; 1997). Chronic hormonal regulation of transporter gene expression has also become evident both *in vivo* and in model cell lines (Reviewed by Rudnick & Clark, 1993).

1.12.1.4. Clinical Significance of 5-HT Transporters.

Blockade of 5-HT plasma membrane transport results in an acute elevation of extracellular 5-HT, resulting in an amelioration of a variety of neuropsychiatric disorders. In fact, neurotransmitter transporter uptake inhibitors are used clinically as anti-depressant drugs for example paroxetine and fluoxetine. However certain drugs of abuse also act to block 5-HT transport for example methylenedioxymethamphetamine (MDMA; 'Ecstasy') and amphetamines. These drugs are substrates for the transporter, and cause transmitter release by a process of transporter mediated exchange. MDMA binds to the transporter at the same site as the selective reuptake inhibitors, and causes the exchange of one molecule of 5-

HT to the outside of the membrane, for one molecule of drug to the inside, leading to large concentrations of synaptic 5-HT. The process of transmitter release is a non-exocytotic process, as the process does not require calcium ions. MDMA also causes degeneration of serotonergic nerve endings by an as yet unidentified process but one that may require dopamine release as well (Rudnick & Wall, 1992). This aspect and the effects of certain uptake blocking drugs will be discussed in more detail in Chapter Six.

1.13 Storage of 5-HT.

5-HT is widely distributed throughout the plant and animal kingdom and storage sites have been located in the pituitary gland, the pineal gland, the pancreas, parafollicular cells of the thyroid gland, the heart, mast cells and the adrenal gland (Saavedra *et al.*, 1974; Koevary *et al.*, 1983; Verhofstadt & Jonsson, 1983; Holzwarth *et al.*, 1984). However the most common sites of storage are in the CNS, the gastro-intestinal tract (GIT) and in platelets.

Neurones containing 5-HT were first identified in the CNS using fluorescent histochemical techniques (Dahlstrom & Fuxe 1964). 5-HTergic neurones are derived from clusters of 5-HT containing cell bodies situated in the brain stem, which project both upwards to various areas in the CNS including the cortex, striatum and hippocampus, and downwards terminating in the spinal cord. 5-HT in the CNS plays many important roles in a range of behavioural patterns including sleep induction, thermoregulation, psychiatric disorders, mood changes, drug dependence and appetite disorders.

In the gut, 5-HT is synthesised and stored in the enterochromaffin cells. The storage granules are located near the base of the enterochromaffin cells.

Almost all circulating 5-HT is contained within platelets. Platelets are small anucleate cells whose main function is hemostasis. Platelets do not synthesise 5-HT, and the majority of 5-HT associated with platelets arises from the enterochromaffin cells of the intestinal mucosa. 5-HT is stored within dense granules in close association with cations, magnesium and calcium, nucleotides and proteins.

Within the adrenal gland 5-HT containing cells have been visualised by immunohistochemistry in various species, including the rat and mouse (Verhofstaad & Jonsson, 1983; Delarue *et al.*, 1988). At the electron microscope level 5-HT appears to be sequestered in chromaffin vesicles within adrenochromaffin cells, indicating that the indoleamine could be released together with catecholamines during splanchnic nerve stimulation (Brownfield *et al.*, 1985). The major metabolite of 5-HT, 5-HIAA, has also been detected in adrenal extracts by high pressure liquid chromatography (HPLC) (Lefebvre *et al.*, 1992). The uptake of 5-HT by adrenaline storing cells in the rat and mouse adrenal medulla has also been observed (Verhofstaad & Jonsson, 1983). In 1992, Lefebvre *et al.*, demonstrated the presence of 5-HT immunoreactive cells in human adrenocortical cells, and concluded that the 5-HT was in fact localised within mast cells found throughout the adrenal cortex. So 5-HT in the adrenal gland could act as a regulator of adrenocortical secretion in a paracrine manner, for example 5-HT released under splanchnic nerve stimulation could contribute to the co-ordinated response of adrenochromaffin and adrenocortical cells during stress conditions.

1.14. Release of 5-HT.

Once stored within the enterochromaffin cells, release of 5-HT can be triggered by a number of stimuli, including vagal nerve stimulation, an increase in transluminal

pressure or a decrease in intestinal pH (Kellum & Jeffe, 1976; Forsberg & Miller, 1983). Upon its release 5-HT is thought to stimulate secretion of pepsin and mucus and inhibit gastric acid production. Also, 5-HT increases the motility of the small intestine and stimulates water and electrolyte secretion. 5-HTergic neurones also provide 5-HT to the gut.

5-HT is released from the platelets by four main mechanisms; exocytosis, exchange, inhibition of transport at the granular membrane and by aminophores and protonophores. These reactions are triggered by a number of stimuli including thrombin, which is involved in the blood clotting reaction. Platelets may act as a circulating store for 5-HT, releasing 5-HT at a site or specific tissue which has no store for 5-HT but does possess 5-HT receptors, such as the thyroid gland and the adrenal (Osim & Wylie, 1983).

5-HT located in mast cells is stored with histamine in large seceretory granules. Compounds that cause exocytosis, including compound 48/80 and some peptides, can release histamine and 5-HT from mast cells simultaneously, and the 5-HT in mast cells may contribute to the content of 5-HT in other organs such as the liver, lung and heart (Jarrott *et al.*, 1975; Gillis, 1985).

1.15. 5-HT Receptors.

5-HT is present in most organisms, ranging from humans to species with primitive nervous systems, for example worms, and the compound itself illicits a wide and varied range of effects both peripherally and centrally. These include constriction and relaxation of smooth muscle, motor activity and psychiatric disorders. This pleiotropy can be explained both by the wide distribution of 5-HT and by the multiplicity of receptors for 5-HT.

Evidence for the existence of serotonergic receptors was first presented by Gaddum & Picarelli in 1957. They described two mechanisms of action of 5-HT in guinea pig ileum, a direct action on the smooth muscle and an indirect action mediated by the release of acetylcholine from parasympathetic nerve endings. As the former mechanism was blocked by dibenzyline (D) and the latter by morphine (M), the excitatory 5-HT receptors on smooth muscle were designated D-receptors and on parasympathetic nerve endings M-receptors. This classification was based purely on pharmacological data and it was not until the advent of radioligands, specific receptor agonists, that a clearer classification was made.

Receptor sites were first observed by Marchbanks (1967) using [^{3}H]-5-HT. Then Farrow & Vunakis (1973) demonstrated that [^{3}H]-LSD binding could be inhibited by 5-HT. The earliest evidence of selective high affinity and saturable binding of [^{3}H]-5-HT to a receptor site was presented almost simultaneously by two groups (Bennett & Snyder, 1976;Fillion *et al.*, 1976). However it was the work of Peroutka & Snyder (1979) that led to the initial classification of 5-HT receptors. They demonstrated that [^{3}H]-5-HT and [^{3}H]-spiperone, a potent 5-HT antagonist, each bound to distinct sites. They named the binding site with high affinity for [^{3}H]-5-HT the 5-HT$_1$ receptor and that with high affinity for [^{3}H]-spiperone the 5-HT$_2$ receptor. In 1981 Pedigo *et al.*, proposed the existence of two 5-HT$_1$ receptor sites, 5-HT$_{1A}$ which exhibited a high affinity for inhibition of [^{3}H]-5-HT by spiperone, and 5-HT$_{1B}$ which exhibited a low affinity for inhibition of [^{3}H]-5-HT by spiperone. A third 5-HT$_1$ receptor was proposed based on the high affinity for inhibition of [^{3}H]-5-HT by mesulergine, termed 5-HT$_{1C}$ (Pazos & Palacios, 1985). Then, various studies with [^{3}H]-ketanserin and [^{3}H]-8-OH-DPAT, by several groups, led to further 5-HT receptor sites being proposed, which differed from the existing sites.

It thus became necessary to reconcile data from functional studies with those from radioligand studies, and provide a common system of nomenclature for the 5-HT receptors identified from the studies performed. In 1986 Bradley *et al.*, proposed a classification system for the three major serotonergic receptor classes, 5-HT_1-like, 5-HT_2 and 5-HT_3. 5-HT_1 -like receptors, described the group of receptors having a high affinity for 5-HT and 5-carboxamidotryptamine and antagonised by methiothepin and methysergide. 5-HT_2 receptors were the initial D-receptor subtype, described by Gaddum & Piccarelli (1957). These receptors mediate a variety of peripheral actions of 5-HT and correspond to the cortical binding sites described by Peroutka & Snyder (1979), having a low affinity for 5-HT and a high affinity for particular serotonergic antagonists such as ketanserin, methysergide, mianserin and metergoline. 5-HT_3 receptors correspond to the M-receptor subtype, and are present in peripheral neurones that mediate the depolarising actions of 5-HT, characterised by their high affinity for cocaine derivatives.

Since this classification scheme was proposed the rapid and extensive advances in development and application of molecular biology techniques has led to the discovery of many new 5-HT receptors, prompting a number of new proposals for a new classification system (Frazer *et al.*, 1990; Peroutka, 1990). Now the classification of 5-HT receptors is based on operational (specific agonists, antagonists and radioligands), structural (molecular biology techniques) and transductional (second messenger systems) criteria. So with the use of these essential data, a "fingerprint" classification can be utilised to identify distinct receptors.

1.15.1. 5-HT_1 Receptors.

These receptors are characterised by a high affinity for 8-OH-DPAT, a moderate affinity for methiothepin, methysergide and quipazine, and a low affinity for

ketanserin, ICS 205-930 and MDL 72222. At least five 5-HT_1 receptor sub-types are now recognised, namely 5-HT_{1A}, 5-HT_{1B} (formerly 5-$HT_{1D\beta}$), 5-HT_{1D} (formerly 5-$HT_{1D\alpha}$) 5-HT_{1E} and 5-HT_{1F}. All are seven transmembrane, G-protein coupled receptors (G_i or G_o), comprising between 365 and 422 amino acids and displaying an overall sequence homology of 40%. These receptors are linked preferentially to the inhibition of adenylate cyclase.

1.15.1.1. 5-HT_{1A} Receptors.

Pedigo *et al.*, (1981) demonstrated that spiperone could be used to discriminate between two different subclasses of 5-HT_1 sites, that is 5-HT_{1A} having a high affinity for spiperone and 5-HT_{1B} having a low affinity. Gozlan *et al.*, in 1983, demonstrated that [^{3}H]8-OH-DPAT bound selectively to the 5-HT_{1A} site. Since these early studies many more selective ligands have become available including 5-MeO-DPAT, buspirone and isapirone. The high affinity of 8-OH-DPAT allowed the affinity purification of 5-HT_{1A} receptors from rat hippocampus (El Mestikawy *et al.*, 1989). A selective antagonist at this receptor is WAY 100635 (Fletcher *et al.*, 1996).

These receptors are located mainly in the hippocampus, septum and amygdala (the limbic system) areas of the CNS, areas concerned with mood and anxiety. Also, to a lesser extent, these receptors are also found in the dorsal and median raphe nuclei where they govern feeding, hyperphagia, male rat sexual behaviour, locomotion and thermoregulation (Hillegaart, 1991). Animals given selective agonists suffer from a stereotypic "5-HT syndrome". In humans this syndrome results in confusion, nervousness, hyper-reflexia, restlessness, hypomania, excitation, myoclony and shivering (Sternbach, 1991). Postsynaptic 5-HT_{1A} receptors are thought to mediate this effect (Tricklebank, 1987). On raphe neurones the receptors are predominantly somatodendritic autoreceptors. Those on serotonergic fibres in the hippocampus are post synaptic (Verge *et al.*, 1986). In both cases they mediate neuronal

inhibition and 5-HT synthesis and release. Northern blot analysis of mRNA from foetal human tissues demonstrated the presence of 5-HT_{1A} receptors peripherally in lymphatic tissues, gut, muscle and kidney, but not in lung, heart, adrenal and placenta (Kobilka *et al.*, 1987). Clinically, selective 5-HT reuptake inhibitors are thought to act on these receptors to produce their antidepressant effects (Blier & De Montigny, 1987).

Human and rat 5-HT_{1A} receptors have been cloned. The receptor consists of a monomeric chain, containing approximately 420 amino acids, characterised by a long third intracellular loop and a short carboxy terminal domain.

5-HT_{1A} receptors can activate several different second messenger systems within the same cell, depending on the G-proteins and effectors present, effecting numerous target proteins and cellular functions. Preferential coupling is to the inhibition of adenylate cyclase via the pertussis-toxin sensitive protein G_i. In HeLa cells, stimulation of PLC and an increase in intracellular calcium was also observed (Fargin *et al.*, 1989; Middleton *et al.*, 1990). In chinese hamster ovary cells, PI hydrolysis was stimulated and also intracellular calcium was increased, in addition to inhibition of adenylate cyclase (Raymond *et al.*, 1992). In the hippocampus, the receptors were found to modulate K^+ channels, via a pertussis-toxin sensitive G-protein which does not involve cAMP, DAG or IP_3 (Andrade *et al.*, 1986; Colino & Halliwell, 1987). In atrial myocytes, the activation of K^+ channels was also observed (Karschin *et al.*, 1991), and in 1993, Dascal *et al.*, identified a cDNA which may correspond to the 5-HT_{1A} activated K^+ channel observed previously in the hippocampus.

1.15.1.2. 5-HT_{1B} Receptors.

5-HT_{1B} receptors were first defined in rat brain (Pedigo *et al.*, 1981) as a component of the high affinity [^{3}H]-5-HT binding sites with a lower affinity for

spiperone than 5-HT_{1A} receptors. Binding sites with similar pharmacological properties have subsequently been described in other rodent species, including the mouse and hamster (Hoyer & Middlemiss, 1989). This receptor originally appeared to be specific to rats and some other rodents. However Hoyer & Baddeke, (1993), characterised the amino acid sequence of this receptor and it was found to be 93% identical overall and 96% identical within the transmembrane domains with that of the 5-$HT_{1D\beta}$ receptor, a close homologue found in higher species, with similar functions and distribution. The difference between these two receptors is attributed to a single amino acid mutation within the transmembrane spanning region (Hoyer & Boddeke, 1993). These receptors are now classified as species homologues of the same receptor, and called h5-HT_{1B} (formerly 5-$HT_{1D\beta}$) and r5-HT_{1B}, with the prefix h denoting human and the prefix r denoting rat. Cyanopindolol, 5-carboxamidotryptamine and RU24969 are all potent non-selective agonists at the receptor, and the indole analogue CP93,129 appears to be the first selective agonist at the receptor site.

These receptors are found mainly in the substantia nigra and basal ganglia, where they function either as autoreceptors on serotonergic projections to the cortex, controlling 5-HT release or as hetero-receptors modulating release of acetylcholine and glutamate (Maura & Raiteri, 1986). Peripherally they inhibit the release of noradrenaline (Molderings & Gothart, 1990), and mediate vascular smooth muscle contraction (Craig & Martin, 1993). They also stimulate the release of prolactin in the rat (Van De Kar *et al.*, 1989). Centrally 5-HT_{1B} receptors may play an important role in modulating the release of other neurotransmitters. The receptors in the rodent may play roles in temperature regulation, stress, feeding and sexual behaviour (Tricklebank *et al.*, 1986; Bendotti & Samanin, 1987; Martin & Peuch, 1991). However more specific agonists and antagonists are needed to provide conclusive evidence to indicate a role for 5-HT_{1B} receptors in these behavioural states.

The rat receptor is a single protein of 386 amino acids, and is linked preferentially to the inhibition of adenyate cyclase. The human receptor is a single protein of 390 amino acids, linked to inhibition of adenylate cyclase (Mengod *et al.*, 1994).

1.15.1.3. 5-HT_{1D} Receptors.

The existence of the 5-HT_{1D} receptor was first described by Heuring & Peroutka (1987) in bovine brain, on the basis of binding studies with [^{3}H]-5-HT in the presence of drugs masking 5-HT_{1A}, 5-HT_{1B} and 5-HT_{1C} subtypes, the remaining binding was termed 5-HT_{1D}. The receptor has a high affinity for 5-HT, 5-methoxytryptamine, 5-carboxamidotryptamine, metergoline and ergometrine. None of these compounds are selective, although a number of potent and relatively selective 5-$HT_{1B/D}$ receptor agonists have been characterised, including sumatriptan, CP-122288, MK 464, GR 46611 and SKF 99101H.

In the CNS of various species the receptor is present in highest density in the substantia nigra, basal ganglia and striatonigral pathway (Waeber *et al.*, 1990). Receptors are also present in the hippocampus, cortex and raphe. Receptor mapping using autoradiography and mRNA hybridisation in rat brain indicated that 5-$HT_{1D\alpha}$ receptors are co-localised with 5-HT_{1B}, but at lower densities (Bruinvels *et al.*, 1991). They function as autoreceptors on cortical nerve terminals and are also located postsynaptically on non-serotonergic terminals, modulating acetylcholine and glutamate release (Raiteri *et al.*, 1986). In peripheral tissues 5-HT_{1D} receptors are involved in neuropeptide release from guinea pig sensory afferent neurones and mediate endothelium-dependent vasorelaxation in some blood vessels (Buzzi *et al.*, 1991). With the advent of more specific ligands the roles of these receptors physiologically will become more apparent.

Human genes encoding two 5-HT_{1D} receptors have been described, 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ and as described previously the 5-$HT_{1D\beta}$ subtype is a non-rodent homologue of the 5-HT_{1B} receptor (Adham *et al.*, 1992). The receptors are single proteins comprising 377 and 390 amino acids respectively. The 5-HT_{1D} (formerly 5-$HT_{1D\alpha}$ exhibits 63% overall structural homology with the 5-HT_{1B} (formerly 5-$HT_{1D\beta}$) and 77% amino acid sequence homology within the seven transmembrane domains. The receptor is negatively linked to adenylate cyclase.

1.15.1.4. 5-HT_{1E} Receptors.

The 5-HT_{1E} receptor was first discovered in human cerebral cortex homogenates as 5-CT-insensitive [^{3}H]-5-HT recognition sites (Leonhardt *et al.*, 1989). Again no selective ligands are available for studying these receptor sites exclusively, they are characterised by having high affinity for [^{3}H]-5-HT binding that is displaced by 5-carboxamidotryptamine and ergotamine with relatively low affinity. The lack of selective ligands has not allowed specific physiological roles for this receptor site to be elucidated. Studies on the distribution of these sites in the CNS have shown a similar distribution to that seen with 5-HT_{1D} receptors, although the two receptor sites exist in various proportions in different areas (Beer *et al.*, 1992). This receptor is concentrated within the caudate putamen, amygdala, frontal cortex and globus pallidus.

The human receptor encodes a single protein of 365 amino acids, with seven transmembrane domains, and sharing approximately 40% homology with other 5-HT_1 receptors (McAllister *et al.*, 1992). When expressed in murine LM (tk-) cells and HEK293 cells the receptor is coupled to inhibition of adenylate cyclase (McAllister *et al.*, 1992).

1.15.1.5. 5-HT_{1F} Receptors

Amlaiky *et al.* (1992), first cloned, in the rat, a new 5-HT_1 receptor site of 367 amino acids, which they termed 5-$HT_{1E\beta}$ as the site had 70% sequence homology with the 5-HT_{1E} receptor. Subsequently the receptor was cloned in human tissue and termed 5-HT_{1F} due to the unique pharmacological profile as compared to other 5-HT_1 receptors, that is a high affinity for 5-HT, sumatriptan, methysergide and methylergonovine, with low affinity for 5-carboxamidotryptamine (Adham *et al.*, 1993). Sumatriptan in fact has almost equal affinity for this receptor as for the 5-$HT_{1B/1D}$ receptor, and thus it has been hypothesised that this receptor may be a target for drugs with antimigraine properties (Adham *et al.*, 1996). LY 334370, is the first selective agonist for this receptor (Wainscott *et al.*, 1996).

In the CNS of the rat the receptor site is found in the cortex, the dorsal raphe and the hippocampus, and in the mouse it is also present in the striatum, thalamus and hypothalamus. The presence of this receptor in the dorsal raphe suggests that it may act as an autoreceptor. Peripherally it is found in the uterus and mesentery.

The receptor preferentially couples to the inhibition of adenylate cyclase, however, when expressed in LM (tk-) cells is coupled to IP_3 formation and a rise in intracellular calcium, and in NIH-3T3 cells the receptor is coupled to inhibition of cAMP formation (Adham *et al.*, 1993).

Recently, a further receptor site belonging to the 5-HT_1 family has been described. Castro, *et al.*, (1997), reported the existence of a new 5-HT1G sub-type in mammalian brain. This site expressed high affinity for 5-carboxamidotryptamine, and is regulated by G-proteins. Due to its unique pharmacological profile, they have putatively called this site 5-HT_8.

1.15.2. 5-HT_2 Receptors

The 5-HT_2 receptor was first described as a site that bound [^{3}H]-spiperone with high affinity and [^{3}H]-5-HT with low affinity (Leyson *et al.*, 1978). It is now known that there are at least three distinct receptor subtypes in this receptor class, that is 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C}. The 5-HT_{2C} receptor was originally termed the 5-HT_{1C} receptor, but the second messenger characteristics and amino acid sequence of this receptor share more in common with the 5-HT_2 receptor class (Hoyer *et al.*, 1993). The receptors are characterised by their high affinity for antagonists such as cyproheptadine, ketanserin, mesulergine, methysergide and metergoline. The receptors exhibit approximately 60% sequence homology, and each is a seven transmembrane, G-protein linked receptor. Unlike the 5-HT_1 receptor site the 5-HT_2 site possesses introns and exons and is coupled preferentially to phospholipase C. Receptor activation thus leads to stimulation of PI metabolism and an increase in IP_3 production.

1.15.2.1. 5-HT_{2A} Receptors.

This is described as the classical 5-HT_2 receptor, widely distributed throughout the CNS and the periphery. The site has high affinity for ketanserin, ritanserin, spiperone, mesulergine and mianserin, although no compound has been shown to be truly selective for this receptor.

In peripheral tissues activation of these receptors leads to blood vessel contraction, contraction of tracheal smooth muscle, bronchoconstriction, platelet aggregation and an increase in capillary permeability (Leysen *et al.*, 1984). They are also involved in modulating the release of other neurotransmitters including vasopressin and dopamine (Muramatsu *et al.*, 1988; Rittenhouse *et al.*, 1990). In the CNS autoradiographic studies demonstrated the presence of this receptor in the cortex, hippocampus, hypothalamus and caudate nuclei (Pazos *et al.*, 1987). Although a role for these receptors centrally remains unclear, in rodents 5-HT_{2A} agonists evoke

a stereotypical behaviour comprising head twitch and wet dog shakes, so the receptors may be involved in motor function. Sharpley *et al.*, (1990), have also reported a role in sleep regulation. In both peripheral and central neurones, the 5-HT_{2A} receptors modulate neuroexcitation and have been associated with the transmission of nociceptive stimuli. The receptors are involved in several disorders including depression, schizophrenia, Parkinson's disease and Alzheimer's disease.

In 1988, Pritchett *et al.*, by screening a rat brain library, isolated a cDNA clone carrying cDNA homologous to the 5-HT_{1C} receptor. After transfection in embryonic human renal cells, this clone produced receptor sites having characteristics of 5-HT_2 receptors, although 5-HT itself had a higher affinity for the cloned receptor. Julius *et al.*, (1990), cloned this gene from rat brain and corrected the published sequence of Pritchett *et al.*, (1988) by one nucleotide. The human receptor was cloned by Kao *et al.*, (1989), and corresponds closely to the rat receptor site with a high degree of homology ~90%. The receptor protein consists of 471 amino acids. The receptor shares an overall sequence identitiy of 53% with the 5-HT_{2C} receptor (80% within the transmembrane domains), and 43% identitiy with the 5-HT_{2B} receptor. The 5-HT_{2A} receptor is coupled in the main to IP_3 formation, although the stimulation of phospholipaseA_2, inducing the release of arachidonic acid in hippocampal and cortical neurones has been demonstrated (Felder *et al.*, 1990).

1.15.2.2. 5-HT_{2B} Receptors.

Foguet *et al.*, (1992), first described this receptor type in rat stomach fundus, where it mediates contraction of smooth muscle. The compounds methysergide, metergoline and LY53857, display high affinity, whereas ketanserin and spiperone display low affinity for this receptor. SB 204741 has been reported to be the first selective 5-HT_{2B} antagonist with approximately 100 fold selectivity over the 5-HT_{2C} and 5-HT_{2A} sites (Baxter *et al.*, 1995).

mRNA for the 5-HT_{2B} receptor has been detected in rat heart, lung, kidney, G.I.T. and discrete regions of the brain (Foguet *et al.*, 1992). A similar distribution was observed for the mouse homologue (Loric *et al.*, 1993). A role in development has been suggested after mRNA for the receptor was found to decline in foetal small and large intestine. In man, 5-HT_{2B} receptor mRNA was found to be expressed in low levels within the brain, and at much higher levels within placenta, lung, liver, kidney, heart, intestine and stomach (Baxter *et al.*, 1995).

Only the rat (479 amino acids), human (479 amino acids) and mouse (504 amino acids) receptor sites have been cloned, exhibiting approx. 50% sequence homology with the 5-HT_{2A} and 5-HT_{2C} receptor sites (Foguet *et al.*, 1992; Wainscott *et al.*, 1993). Expressed in mammalian cells 5-HT_{2B} receptors stimulate the production of IP_3, also in the mouse activation of this receptor causes a rapid influx of chloride ions, this effect may be secondary to IP_3 activation (Loric *et al.*, 1993).

1.15.2.3. 5-HT_{2C} Receptors.

Originally this receptor was termed 5-HT_{1C}, but the second messenger characteristics and amino acid sequence clearly demonstrated that this receptor type belonged to the 5-HT_2 family. The receptor has high affinity binding for [^{125}I]LSD, which can be displaced by mianserin, 5-HT and spiperone. The novel ligands, SB 200646A and SB 206553, have been reported to be selective 5-$HT_{2C/2B}$ receptor antagonists with 50-100 fold lower affinity for the 5-HT_{2A} receptor and other sites (Kennett *et al.*, 1996). Two truly selective compounds have also been described, SB 242084, displaying over 100 fold selectivity over other sites and, RS-102221, also displaying over 100 fold selectivity (Bonhaus *et al.*, 1997; Kennett *et al.*, 1997).

Autoradiography and *in situ* studies demonstrate high receptor densities in the choroid plexus and lower receptor densities in the limbic system, basal ganglia, hypothalamus and hippocampus (Molineaux *et al.*, 1989). The main function of this receptor is in the regulation of exchanges between the CNS and cerebrospinal fluid (Hartig, 1989). Tsutsumi & Sanders-Bush demonstrated that the production of transferrin by choroid plexus epithelial cells was regulated by 5-HT_{2C} receptors. These receptors may also play roles in locomotion, eating disorders, anxiety and migraine (Fozard, 1992). A potential role in protooncogenes has also been demonstrated (Julius *et al.*, 1989). In the periphery, the receptor mediates endothelium-dependent vasorelaxation (Glusa, 1992).

The rat and human receptors have been cloned. The receptor is a monomeric protein belonging to a large multigenic family characterised by seven transmembrane spanning domains and linked to a G-protein, consisting of 460 amino acids (rat) and 458 amino acids (human). The receptor is coupled to activation of IP_3 with a concomitant increase in intracellular calcium levels (reviewed by Hoyer & Martin, 1997).

1.15.3. 5-HT_3 Receptors.

The 5-HT_3 receptor subtype is unique among other 5-HT receptors in that it is not linked by a G-protein to its effector system, but in fact it forms a ligand-gated ion channel analogous to the GABA, glycine and nicotine receptors.

They are present in neurones in many tissues in the periphery and in the brain. Peripherally the receptors are present both pre- and postsynaptically, in both branches of the autonomic system and on afferent and enteric neurones (Fozard, 1984). In the GIT 5-HT_3 activation modulates secretion and motility (Costall *et al.*, 1990). Other consequences of 5-HT_3 activation in the periphery are cardio-activation and inhibition, vasodilatation, pain and initiation of the vomiting reflex.

This latter effect is thought to underlie the nausea side effect of cancer chemotherapy, and has led to the use of selective 5-HT_3 receptor antagonists as antiemetic agents. In the CNS the receptors have a wide distribution, located in the area postrema, hippocampus, dorsal vagal complex and substantia gelatinosa. The cerebellum is devoid of these receptors. The receptors modulate neurotransmitter release centrally, facilitating the release of dopamine, GABA and 5-HT and inhibiting the release of acetylcholine and noradrenaline. Behaviourally, these receptors play a role in anxiety, depression, memory and the effects of tolerance (Barnes *et al.*, 1992).

A cDNA encoding a single subunit of this receptor has been isolated from a mouse neuroblastoma cell line (Maricq *et al.*, 1991). The predicted protein comprises 487 amino acids and contains four hydrophobic transmembrane spanning domains. Receptor activation triggers rapid opening of a transmembrane channel resulting in an increase in Na^+/K^+ conductance and a subsequent rapid influx of extracellular calcium (Peters *et al.*, 1991). The response, which can rapidly desensitise, is G-protein independent and insensitive to pertussis toxin. Stimulation of IP_3 production in response to receptor activation has also been described (Ashby *et al.*, 1990), implying coupling to a G-protein, however the very fast kinetics of channel activation suggest that this is more probably secondary to ionic events, such as elevation of cytosolic calcium.

1.15.4. 5-HT_4 Receptors.

The 5-HT_4 receptor was first described by Dumuis *et al.*, (1988), in the CNS as a receptor positively coupled to adenylate cyclase, displaying an unusual pharmacological profile. The first agonists reported for this receptor were the substituted benzamide and benzimidazolone derivatives, for example, zacopride, cisapride, BIMU 1 and BIMU 8 (Dumuis *et al.*, 1992). However these compounds also displayed various affinities for the 5-HT_3 receptor. Tryptamine derivatives

including 5-methoxytryptamine, 5-carboxamidotryptamine and tryptamine are relatively selective agonists at this receptor. There are several selective antagonists for this receptor, including, GR 113808, RS 23597-190, RS 39604 and DAU 6285.

In various species the 5-HT_4 receptor has a wide distribution. Centrally these receptors are located in the hippocampus, striatum, substantia nigra and olfactory tubercle. No receptors appear in the cerebellum and frontal cortex. In the periphery 5-HT_4 receptors mediate relaxation and contraction of gastrointestinal muscle (Craig & Clarke, 1990), an increase in cardiac rate and force of contraction (Kaumann, 1991) and an increase in steroid release from the human and frog adrenal glands (Lefebvre *et al.*, 1993).

The 5-HT_4 receptor has been cloned from rat, mouse and human brain and heart RNA by the reverse transcriptase-polymerase chain reaction (Gerald *et al.*, 1995; Claeysen *et al.*, 1996; 1997). Two different cDNA's were isolated, named 5-HT_{4a} and 5-HT_{4b}. These splice variants encoded proteins of 387 and 406 amino acids respectively, containing 96.1% identical peptide sequences and diverging in the second half of the carboxyl terminus tails at position 360 where there appears to be an intron. Another splice variant 5-HT_{4c} has been cloned from rat, mouse and human brain, encoding a protein of 372 residues (Claeysen *et al.*, 1998). This receptor type contains a short third intracellular loop (45 amino acids) and long carboxyl termini (70; 89 amino acids). Activation of these receptors centrally leads to elevation of cAMP, a decrease in potassium conductance and a stimulation of local release of acetylcholine (Dumuis *et al.*, 1988; Boddeke & Kalkman, 1992).

1.15.5. 5-ht_5 Receptors.

The 5-$_{ht5A/5B}$ receptors display high affinity binding to [^{125}I]-LSD in certain cell types, that is displaced by methysergide, methiothepin and ergotamine (Plassat *et*

al., 1992; Matthes *et al.*, 1993). 5-HT, 5-carboxamidotryptamine, 8-OH-DPAT and sumatriptan all act at this receptor.

The 5-ht_{5A} receptor is expressed exclusively within the CNS. 5-ht_{5A} mRNA has been detected via northern blot analysis and quantitative PCR in mouse brain, spinal cord and cerebellum, but not in kidney, liver, spleen or heart (Plassat *et al.*, 1992). *In situ* hybridisation experiments show the presence of this receptor in mouse cerebral cortex, hippocampus, cerebellum, habenula and olfactory bulb. Northern blot analysis of rat brain revealed the presence of this subtype in many areas including hippocampus, hypothalamus, cortex, thalamus, pons, striatum and medulla (Erlander *et al.*, 1993). Studies utilising a specific 5-ht_{5A} antibody have suggested that this receptor is mainly expressed in glial cells and that this receptor may act as a terminal autoreceptor within the mouse frontal cortex (Erkander *et al.*, 1993).

Northern blot analysis of mouse tissue demonstrated that the 5-ht_{5B} receptor is not present in brain, kidney, heart, lung and intestine, *in situ* hybridisation on mouse brain sections located the receptor exclusively in the hippocampus, habenula and the dorsal raphe (Matthes *et al.*, 1993). In the rat the receptor is present in the hippocampus, dorsal raphe, olfactory bulb, habenula and subiculum. No receptor activity was located in rat liver, muscle, lung, heart, kidney and spleen (Wisden *et al.*, 1993).

Potential physiological roles for these receptors have not been investigated. As the transductional and operational characteristics of this receptor are still being investigated the lower case appellation is presently used to define these gene products.

The 5-ht_{5A} receptor has been cloned from mouse, rat and human tissues, whilst the 5-ht_{5B} receptor has been cloned from mouse and rat (Plassat *et al.*, 1992; Erlander *et al.*, 1993; Wisden *et al.*, 1993). The receptors contain 357 amino acids (5-ht5Amouse) and 370 amino acids (5-ht5Bmouse) and exhibit a structural homology of 88%. The receptors are seven transmembrane G-protein coupled receptors, and at present the second messenger system has not been fully identified. In cells expressing the cloned 5-ht_{5A} site, the receptor was shown to be negatively linked to adenylate cyclase via the G_i-protein (Carson *et al.*, 1995). When the 5-ht_{5B} receptor was expressed in COS-1 cells a fraction of the binding sites were displaced by GTP analogues suggesting that this receptor couples to G proteins in these cells (Wisden *et al.*, 1993). So this receptor may interact with an effector system other than adenylate cyclase or PLC.

1.15.6. 5-HT_6 Receptors.

This receptor has pharmacological characteristics similar to the 5-HT_1 receptor, with high affinity for methiothepin and 5-methoxytryptamine (Monsma *et al.*, 1993). The receptor also displays high affinity for a number of anti-depressant drugs, including amitriptyline, clomipramine and amoxipine and the antipsychotic drugs clozapine and loxapine.

In situ hybridisation studies located this receptor in the rat striatum, olfactory tubercle, cortex and hippocampus (Ruat *et al.*, 1993a). Low levels were expressed in rat hypothalamus and stomach. No signal was detected in medulla, pituitary, retina, thalamus, cerebellum and various peripheral tissues. Northern blot analysis using guinea pig tissues, striatum and olfactory tubercle produced the strongest signal, followed by cerebellum, brain stem, olfactory bulb and substantia nigra. A faint signal was also detected in the adrenal glands (Ruat *et al.*, 1993a). No mRNA was detected in guinea pig ileum, lung, atrium or pituitary (Ruat *et al.*, 1993a). *In situ* hybridisation revealed the presence of 5-HT_6 receptor mRNA in rat olfactory

tubercles and bulb, striatum, nucleus accumbens, hippocampus and cerebral cortex (Ruat *et al.*, 1993a). An *in vivo* role for this receptor has been suggested in neuropsychiatric disorders due to its presence within limbic pathways and its high affinity for certain antipsychotic and antidepressant drugs (Roth *et al.*, 1994)

The 5-HT_6 receptor was cloned from a rat cDNA library and was found to be positively coupled to adenylate cyclase (Montsama *et al.*, 1993). The cDNA encodes a 436 amino acid protein with seven hydrophobic regions, with approximately 40% homology with other 5-HT receptors. The receptor has a short third intracellular loop (50 amino acids), long carboxy tail (120 amino acids) and an intron between the sixth and seventh transmembrane loop, and this indicates a novel 5-HT receptor class. The human gene has also been cloned and exhibits 89% sequence homology with the rat receptor. The receptor is positively linked to cAMP formation

1.15.7. 5-HT_7 Receptors.

The site has high affinity for 5-carboxamidotryptamine, 5-methoxytryptamine, methiothepin, mesulergine, 8-OH-DPAT and a lower affinity for ketanserin (Jakeman *et al.*, 1993). [^{3}H]-SB-269970 is the first selective antagonist radioligand at this site (Thomas *et al.*, 2000).

Northern blot analysis of rat tissue located highest levels of mRNA in hypothalamus, thalamus, brainstem and hippocampus, with lower levels in cortex, striatum, olfactory bulb and tubercle (Shen *et al.*, 1993). Faint signals were located in the spleen, stomach and ileum (Ruat *et al.*, 1993b; Shen *et al.*, 1993). The expression of this receptor in various hypothalamic nuclei has led to the suggestion that this receptor may be important in circadian rhythms (Lovenberg *et al.*, 1993) and may have potential therapeutic roles in depression and schizophrenia (Sleight *et al.*, 1995; Roth *et al.*, 1994). In man, mRNA was also found in the coronary

Table 1.1 : 5-HT Receptor Subtypes.

	5-HT_{1A}	5-HT_{1B}	5-HT_{1D}	5-HT_{1E}	5-HT_{1F}	5-HT_{2A}
Agonists	8-OHDPAT Buspirone Isapirone 5-CT	CP93129 Sumatriptan 5-CT	Sumatriptan GR46611 L694247 5-CT	Ru 24969 5-CT	LY334370 5-CT	α-Methyl5-HT
Antagonists	WAY100135 WAY100635 Spiperone	GR55562 SB242289 Pindolol	GR127935	Methiothepin	Methiothepin	Ketanserin Ritanserin Cyproheptadine MDL11939 MDL100907
Radioligands	[^{3}H]8-OH-DPAT	[^{125}I]Iodo-cyanopindolol	[^{3}H]5-HT	[^{3}H]5-HT	[^{3}H]LSD	[^{3}H]Ketanserin
Second Messengers	cAMP↓ K^+Channel	cAMP↓	cAMP ↓	cAMP↓	cAMP↓	IP_3/DAG

5-HT_{2B}	5-HT_{2C}	5-HT_3	5-HT_4	$5\text{-ht}_{5A/5B}$	5-HT_6	5-HT_7
αMethyl-5-HT BW723C86	αMethyl-5-HT MK 212 mCPP	2-Methyl-5-HT RS56812	RS67333 RS67506 Renzapride 5-MeOT	5-CT LSD	5-CT LSD	5-CT LSD 5-MeOT
SB206553 SB204741 LY266097 SB200646A Ketanserin	SB206553 SB221284 Mesulergine SB200646A Ketanserin	GR65630 MDL72222 CS205-930 Granisetron Zacopride Ondansetron	SB203186 GR113808 SB204070 LY297582 DAU6285 RS23957 SB204070		Methiothepin Amoxipine Clozapine	
	[^{3}H]mesulergine [^{3}H]LSD	[^{3}H]GR65630 [^{3}H]Granisetron [^{3}H]Zacopride	[^{3}H]GR113808	[^{3}H]LSD	[^{3}H]LSD	[^{3}H]LSD
IP_3/DAG	IP_3/DAG	Internal cation channel	cAMP ↑	Unknown	cAMP ↑	cAMP ↑

artery, descending colon, GIT, stomach and ileum (Bard *et al.*, 1993; Schoeffter *et al.*, 1996). The receptor site has been found to be present within the rat adrenal gland (Contesse *et al.*, 1999).

This receptor has been cloned from rat (448 amino acids, Ruat *et al.*, 1993b; Shen *et al.*, 1993; Lovenberg *et al.*, 1993), mouse (448 amino acids, Plassat *et al.*, 1993), human (445 amino acids, Bard *et al.*, 1993) and guinea pig (466 amino acids, Tsou *et al.*, 1994) cDNA libraries. This receptor contains seven transmembrane domains and is coupled to the activation of adenylate cyclase. Structural data indicate that this represents another distinct class of receptors. The receptor possesses two introns in the gene coding region, and possesses various (40%-60%) sequence homology with the other 5-HT receptors, but high interspecies homology (95%). A number of splice variants of both the human ($5\text{-HT}_{7a/7b/7d}$) and rat ($5\text{-HT}_{7a/7b/7c}$) receptor have been identified, which display similar pharmacological and functional characteristics when expressed in cell lines (Jasper *et al.*, 1997; Heidman *et al.*, 1997).

1.16. Aims of this Thesis.

In attempting to clarify a physiological role for 5-HT in aldosterone secretion it is necessary to address a few questions. Is 5-HT able to stimulate aldosterone directly? Does the sensitivity of this aldosterone response to 5-HT increase in states of sodium depletion? If 5-HT does influence aldosterone secretion what is the physiological source of 5-HT reaching the ZG, and can this source also be regulated by salt intake? Are there specific receptors for 5-HT within the rat ZG? Does the 5-HT transporter have a role to play in the ZG?

The main aim of this thesis was to establish a role for 5-HT in the control of aldosterone secretion. 5-HT is well known for its roles as a neurotransmitter and in

the blood clotting cascade, but the steroidogenic action of 5-HT in the adrenal gland is an area of interest that still needs to be defined.

In order to gain more knowledge on the direct effects of 5-HT on aldosterone, structure activity studies were performed using a range of indoleamines in isolated rat zona glomerulosa cells. These studies, described in Chapter 3, tested the potency of several indoleamines, some naturally occurring, relative to 5-HT in order to determine the functional groups on the indole nucleus which are necessary for eliciting biological activity. In conducting these experiments, the hypothesis that 5-HT may not be the only physiological indoleamine that can regulate aldosterone was tested. Following on from this study, experiments were then conducted, utilising a variety of 5-HT agonists and antagonists, in order to characterise pharmacologically the 5-HT receptor type that mediates the aldosterone response to 5-HT within the rat zona glomerulosa. These experiments are described in Chapter 4. Some of these agonists and antagonists were also studied in isolated rat zona glomerulosa cells which had been prepared from rats maintained on different salt diets, in order to test the salt sensitivity of the aldosterone response to 5-HT receptor activation and inhibition. A further aim of the work within this thesis was to identify possible local sources of 5-HT in the rat adrenal gland using both immunohistochemical and biochemical techniques. The major focus was to determine whether significant levels of the enzyme L-AAAD, which can synthesise both 5-HT and dopamine from their respective precursors, 5-HTP and L-DOPA, are present within the adrenal cortex and more specifically the zona glomerulosa. These studies are described in Chapter 5. The hypothesis that adrenocortical L-AAAD could potentially be modulated by salt intake was also tested in the experiments described. The final aim was to investigate whether aldosterone secretion can be affected by drugs that are known to affect the 5-HT transporter and to determine by autoradiography the location of a potential 5-HT transporter within the rat adrenal gland. These studies are described in Chapter 6.

Chapter Two

Materials and Methods.

2.1 Introduction.

This chapter describes the general methodology, specialised techniques and laboratory procedures used throughout these studies. At the beginning of each new chapter a brief review of those techniques relevant to that chapter will be given.

2.1.1. Animal and Controlled Drug Legislation.

A. Animal.

All experimental procedures were carried out humanely via Schedule 1 methodology, described in the Animals and Scientific Procedures Act, 1986. Any procedures not covered by Schedule 1 methodology, were conducted following the authority of a personal licence, controlled by an appropriate project licence.

B. Controlled Drug.

All use of the controlled drug MDMA was carried out following the Misuse of Drugs Regulations Act, 1985, under a project licence held at the University of Edinburgh, Department of Clinical Neuroscience.

2.2 Indoleamine Study.

2.2.1 Isolation Of Zona Glomerulosa Cells.

Female Wistar rats (200-250g), were killed by dislocation of the cervical vertebrae, and the adrenal glands coarsely dissected with their surrounding fat and placed into normal saline. Each adrenal was placed on moistened, hardened filter paper and trimmed of all fat. The capsules and adhering zona glomerulosa were removed following the method of Haning *et al,* 1970. Briefly each adrenal was bisected with a scalpel, leaving a bridge of capsule connecting the two hemiadrenals. When the adrenal was turned onto its cut surface, and the capsule bridge held with forceps, the adrenal was easily decapsulated by applying gentle pressure with the back of a

scalpel blade. The capsules were then placed in normal saline and agitated gently to remove debris. For collagenase digestion the capsules were incubated for 1 hour at 37°C in Krebs-Ringer bicarbonate buffer, previously gassed for 15 minutes with 95% O_2/5% CO_2 and containing 20g/L bovine serum albumin (BSA), 2g/L D-glucose (KRBGA) and 2mg/ml/rat collagenase crude type I. The capsules were disaggregated gently at 20 minute intervals using a wide mouthed 5ml pipette. After 1 hour the dispersed cells were filtered through a 100μ gauze to remove coarse debris. The filtrate was immediately centrifuged at 1000 rpm and 4°C for 15 minutes, causing the cellular suspension to pellet. The supernatant was removed and replaced with 5ml of fresh KRBGA into which the cell pellet was resuspended. This washing procedure was repeated 3 times and after the final wash the supernatant was replaced with 10mls of Medium 199, containing 5g/L BSA and 2g/L D-glucose. After thorough resuspension, the material was filtered through a 56μ gauze, and an aliquot removed for cell counting using a haemocytometer. On average this method produced a cell suspension containing at least 95% zona glomerulosa cells, with less than 5% zona fasciculata cell contamination.

2.2.2. Cell Counting.

20μl of the cell suspension was added to a 20μl aliquot of a 0.2% (w/v) solution of trypan blue, and mixed thoroughly. A small aliquot was placed in the counting chamber of a haemocytometer and allowed to diffuse. The cells were then viewed with a microscope at a 400 fold magnification and those in the outside 4 and centre squares which excluded trypan blue and thus considered viable were counted. The number was then corrected as follows to give the total number of cells.

Total Cells = number counted within the squares x 2 (trypan blue dilution factor) x 20,000 (converts volume of chamber to total volume of cells i.e. 10mls). A typical yield from 4 adult rats, i.e. 8 adrenal capsules was approximately 300,000 cells per 1ml suspension.

2.2.3. Cell Incubations.

Initially 400µl of the cell suspension containing ~ 100,000 zona glomerulosa cells was used per 1ml incubation. However using such a large number of cells per incubation resulted in a large number of animals being used per experiment, so it was decided to try and miniaturise the incubation to 200µl, using only 20,000 zona glomerulosa cells. To ensure that no loss of response resulted in this miniaturisation the two different protocols were compared (see Figure 2.1.).

100µl of the cell suspension, diluted to contain ~20,000 zona glomerulosa cells, was added to eppendorf tubes containing 100µl of Medium 199 supplemented with 5g/L BSA;2g/L D-glucose, in which all the test substances (10^{-10}M-10^{-4}M) had been dissolved to the required final concentration. Control incubations contained zona glomerulosa cells and Medium 199 only, allowing the basal value for aldosterone secretion into the medium to be measured. Each incubation was carried out in triplicate and the dose response to 5-HT carried out at least twice during each individual experiment to allow comparison of aldosterone secretion in response to 5-HT with responses to other compounds. The tubes were mixed thoroughly and incubated for 1 hour at 37°C in a shaking water bath. After 1 hour the tubes were placed on ice to prevent further steroid production and then centrifuged at 15,000g for 2 minutes at 4°C to pellet the cell suspension. The supernatant was decanted and the samples stored at –20°C prior to the measurement of aldosterone secretion into the medium via direct radioimmunoassay (RIA).

2.2.4. Aldosterone Radioimmunoassay.

Aldosterone was measured by direct RIA (Campbell *et al.* 1981). 50µl aliquots from the cell incubation extracts or 50µl aliquots containing a known concentration of aldosterone, for the standard curve, were incubated in triplicate with 200µl of

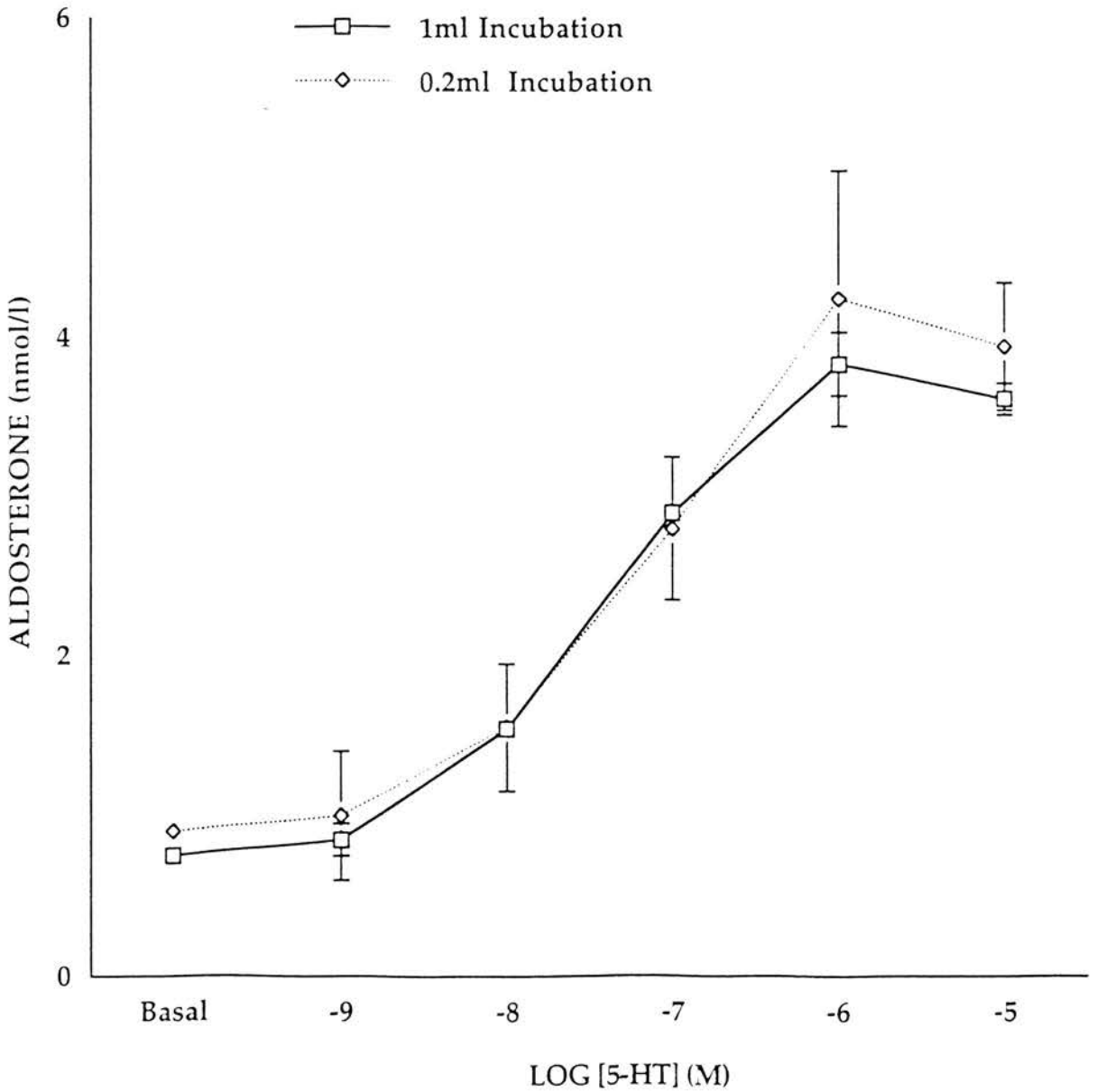


Figure 2.1 Comparison of cell incubations.

phosphate buffer (5g/L BSA) containing ^{125}I-aldosterone (3000-4000cpm) and 402L antibody to aldosterone, diluted to give an initial titre of 1:100,000. This polyclonal antibody was kindly donated by Dr F.A.O. Mendelsohn, Australia. The tubes were incubated overnight at 4°C. Separation of the bound and free radiolabel in each tube was carried out at 4°C by the addition of 0.5ml of dextran coated charcoal followed by centrifugation at 4000rpm for 20 minutes at 4°C. The supernatant was aspirated and the charcoal pellet, containing the free fraction, counted in an LKB multiwell gamma counter.

The aldosterone 402L antibody was raised in a rabbit to aldosterone-3-carboxymethyloxime conjugated to bovine serum albumin by the method of Erlanger *et al.*, (1957). The antibody has the following cross-reactivities; 17-isoaldosterone (25%), tetrahydroaldosterone (20%), 18-hydroxydeoxycorticosterone (0.025%), 18-hydroxycorticosterone (0.025%) and aldosterone-y-lactone (0.02%). The following have less than 0.0001% cross-reactivity, corticosterone, 11-dehydrocorticosterone, deoxycorticosterone, cortisol, cortisone, progesterone, pregnenolone, tetrahydrocorticosterone, tetrahydrocortisol, tetrahydrocortisone, dexamethasone and spironolactone. Using 50pg [1,2,6,7,-^{3}H]aldosterone/assay tube, the assay had a sensitivity of 5pg (Campbell *et al.*, 1981).

The lower limit of detection for the assay was 0.06 nM, and the working range for aldosterone measurement was 0.06 to 32nM. The intra and inter assay co-efficients of variation were 7% and 15% respectively.

2.2.5. Cyclic AMP Radioimmunoassay.

Cyclic AMP was measured by direct RIA developed in this department by Dr I.F.Gow (Harper & Brooker 1975). Prior to RIA, 500µl of the cell incubation was acidified by adding 5µl of 20% (v/v) acetic acid and acetylated, in parallel with

cAMP standards, with 15µl of a solution containing 2 parts triethylamine: 1 part acetic anhydride. 50µl aliquots of the acetylated sample or 50µl of cyclic AMP standards were incubated in triplicate with 200µl of acetate buffer (0.05M, pH 4.8 containing 0.1% BSA) containing ^{125}I tyrosine-methyl-ester succinyl cyclic AMP (^{125}I-cyclic AMP) (3000-4000 cpm) and antisera (R1B6) to cyclic AMP to give an initial dilution of 1:10,000. The tubes were allowed to equilibrate overnight at 4°C. Separation of bound and free radiolabel was carried out using dextran coated charcoal as described in 2.2.4.

The lower limit of detection for the assay was 0.06nM, and the working range of the assay was 0.06nM to 32nM. The intra and inter assay co-efficients of variation were 8% and 16% respectively.

2.2.6. Preparation Of Iodinated Aldosterone.

2.2.6.1. Activation Of Aldosterone-3-carboxymethyl-oxime.

Aldosterone-3-carboxymethyl-oxime (0.29mg) was dissolved in 400µl ethanol and then dried down in a dri-block under nitrogen. This was then redissolved in 50µl of dimethylformamide (DMF), which had been cooled to 10°C. The oxime was maintained at 10°C in a stirred water bath and to the redissolved oxime, 10µl of tributylamine (85µl in 5ml DMF) and 10µl of isobutylchloroformate (45.5µl in 5ml DMF), were added. This solution was then vortexed and kept at 10°C for 20 minutes.

2.2.6.2. Iodination Of Histamine.

10µl of Na^{125}I (equivalent to 1mCi/37MBq), 10µl of histamine (2.2mg in 10ml 0.25M phosphate buffer) and 10µl of chloramine T (50mg in 10ml of 0.25M phosphate buffer) were added to a conical glass tube and vortexed for 15 seconds. 10µl of sodium metabisulphite (120mg in 10ml of 0.05M phosphate buffer) was then added and the solution was vortexed and cooled to 0°C in ice. 280µl of

cooled DMF (10°C) was added to the activated steroid after the 20 minute interval and the solution was vortexed. 50µl of this diluted steroid mixture was then added to the ice cold iodination mixture, and then 10µl of 0.2M NaOH was added. The mixture was then vortexed and kept at 0°C for 1 hour. 1ml of 0.1M HCl and 1ml of redistilled ethyl acetate was then added to the iodination mixture and the mixture then vortexed for three seconds. The upper (organic) layer was then removed, counted and discarded. 1ml 0.1M NaOH and 1ml potassium iodide (100mg in 10ml 0.5M phosphate buffer) were then added and the mixture vortexed before being extracted with 750µl of ethyl acetate as described.

250µl redistilled ethyl acetate was added to the iodination mixture and the mixture was vortexed for 60 seconds. The lower aqueous layer was removed to a second conical glass tube and the organic layer was transferred to a clean glass tube. The aqueous layer was extracted a further two times, with the organic layers being combined.

The iodinated 3-CMO aldosterone was then purified by thin layer chromatography. The organic extract was applied in a thin line along the bottom of a 200x200mm silica gel TLC plate and run in a solvent of toluene:ethanol:acetic acid (75:24:1) for approximately 1 hour. The plate was then removed from the tank and allowed to dry before being placed in a film box and exposed to Kodak X-ray film for one hour. The position of the ^{125}I - 3CMO-aldosterone band was marked on the TLC plate and the silica scraped off carefully. The silica was then transferred via a glass funnel to a glass scintillation vial and the iodinated 3-CMO aldosterone eluted with 3ml ethanol for one hour at room temperature. The ethanol was then filtered through a glass pasteur pipette plugged with a small piece of cotton wool and containing 2cm celite to remove the silica. The column was washed with 3x1ml ethanol. The final ^{125}I -3CMO-aldosterone product was stored at – 20°C.

2.3. 5-HT Agonist And Antagonist Study.

2.3.1.Isolation Of Zona Glomerulosa Cells.

As in section 2.2.1.

2.3.2.Cell Counting

As in section 2.2.2.

2.3.3.Static Cell Incubations

Incubations were set up as in section 2.2.3. Full dose responses (10^{-10}M-10^{-4}M) to all 5-HT agonists were set up, in triplicate, in individual experiments. For the 5-HT antagonists dose responses to each antagonist were set up, in triplicate, in the presence of 10^{-7}M 5-HT, i.e. a sub-maximal concentration of 5-HT. The incubations were treated and stored as in section 2.2.3.

2.3.4.Aldosterone Radioimmunoassay

As in section 2.2.4.

2.3.5. Cyclic AMP Radioimmunoassay.

As in section 2.2.5.

2.4. L-Aromatic Amino Acid Decarboxylase (L-AAAD) Study.

2.4.1. Salt Diets.

12 female Wistar rats (200-250g) were housed in individual metabolic cages for 1 week to equilibrate with the environment. They were then maintained on one of the following diets for one week:-

1. Control diet	Wholemeal flour containing 1% NaCl
2. Low sodium diet	Wholemeal flour containing 0% NaCl
3. High sodium diet	Wholemeal flour containing 3% NaCl

Urine was collected from each animal two days prior to and daily for the week of the salt diets. The pH of the urine was maintained between pH 2-3 by addition of 0.5 ml of 5M HCl into each urine pot. This is essential to preserve all samples in the reduced state prior to measurement via HPLC. 5-HT, dopamine, L-DOPA and 5-HIAA were all measured in the urine samples by HPLC performed at the RIE by Neil Johnston. Basically, 0.5g of activated aluminium oxide was added to a series of tubes and washed with 5ml EDTA (0.2M). 5ml test urine samples and 0.5ml internal standards were then added to the tube and mixed thoroughly and then centrifuged at 4°C/1000RPM for 2-3 minutes. The supernatant was siphoned off and the deposit washed three times with deionized water. 2ml of 0.2M perchloric acid was then added, to elute the amines to be measured, and mixed thoroughly and centrifuged at 4°C/1000RPM for 2-3 minutes. Porex filters were then added to all sample tubes and pressed down until all the supernatant was vented through the filter. The supernatant was then decanted and the sample stored at –40°C until measurement by HPLC using electrochemical detection.

After the appropriate time period the animals were killed by decapitation. Blood samples were obtained, the adrenal glands and kidneys were removed with their surrounding fat and placed in pots of saline. The capsules, removed following the method of Haning *et al.* (1970), with adhering zona glomerulosa were incubated with increasing concentrations of 5HTP or L-DOPA (10^{-6}M-10^{-3}M), all in the presence of 100 µl 10^{-4} M pargyline, to inhibit monoamine oxidase, in a total volume of 1ml of KRBGA. If the capsules were incubated with L-DOPA, the tissue was prestimulated with 10^{-5}M 5-HT. In each set of incubations one incubation contained 10^{-4}M carbidopa, to block the enzyme L-AAAD. The tubes were then incubated in a shaking water bath at 37°C for 1 hour.

After the incubation, the supernatant was removed into microtubes and stored at -20°C for subsequent measurement of aldosterone by radioimmunoassay (RIA) and L-DOPA, dopamine, 5HTP, 5HT and 5HIAA by high pressure liquid chromatography (HPLC). The tissue was retained for protein assay.

2.4.2. Isolation of zona glomerulosa cells and zona fasciculata cells.

12 female Wistar rats (200-250g) were maintained on normal, low and high salt diets for 1 week as described above, in free running cages. After the appropriate time period the animals were killed by decapitation. Blood samples were obtained and the adrenal glands were dissected as described above. The capsule and adhering zona glomerulosa were treated as in section 2.2.1 to prepare a suspension of zona glomerulosa cells. The inner cores of the adrenal glands, containing the zona fasciculata cells were also treated as in section 2.2.1., the cell suspension was only mechanically dispersed at the end of the 1 hour incubation. The collagenase digestion destroyed the medullary component of the adrenal cores. After the final spin, the zona glomerulosa cells from each diet and the zona fasciculata cells from each diet, were resuspended in 3.2ml of medium 199 i.e. to correspond roughly to the amount of zona glomerulosa/fasciculata cells from each capsule/core. Incubations were set up with increasing concentrations of 5HTP or L-DOPA (in the presence of 5-HT) as described above, and contained the following:

500 µl Medium 199 containing 10^{-4}M pargyline and +/- 10^{-4}M carbidopa.

100 µl Agonist

400 µl of cells which equated to roughly 1 capsule

The tubes were mixed gently by vortex, then transferred to a shaking water bath at 37°C and incubated for 1 hour. At the end of the incubation, the tubes were centrifuged at 10,000 rpm and 4°C for 15 minutes, causing the cells to pellet. The

supernatant was decanted and stored at -20°C until measurement by HPLC or RIA, while the cell pellet was kept for protein assay.

2.4.3. Treatment of blood samples.

225 µl of trunk blood was transferred to a tube containing 25 µl of ice cold ethylenediaminetetraacetic acid (EDTA) inhibitor (27mmol/l). This was vortexed and then centrifuged at 10,000g for 15 minutes at 4°C. The supernatant was removed and stored at -20°C until measurement of aldosterone by RIA.

20 µl of the remaining whole blood was added to 180 µl of a cocktail containing thrombin (1100 units/l), chlorimipramine (1.1 mmol/l), and pargyline (11.1 mmol/l). This was left at 4°C for 2 hours to allow complete release of 5-HT by the platelets and then centrifuged at 10,000g for 15 minutes at 4°C. The supernatant was removed and stored at -20°C before measurement of 5HTP, 5HT and 5HIAA by HPLC.

2.4.4. HPLC for measurement of L-DOPA, Dopamine, 5HTP, 5HT and 5HIAA.

All compounds were measured in each sample using a high pressure liquid chromatography (HPLC) with electrochemical detection (Gow *et al.*, 1987). The HPLC system consisted of a Waters 510 pump, a U6K injector, a Waters 150x3.9 mm µBondapak C_{18} reversed-phase analytical column, a BAS LC-4A electrochemical detector with a single glassy carbon electrode and a Waters data module.

Each sample was thawed and prepared on the day of sampling. 400 µl of the sample was added to 50 µl of the internal standard N- methyl5-HT or N-methyldopa (10µm) and 50 µl of 15% perchloric acid/2 mM cysteine in a microtube, vortexed and stored at 4°C for 15 minutes to allow complete deproteinisation. Tubes were

then centrifuged at 10,000g at 4°C for 15 minutes. Samples were injected individually in a volume of 25 µl. Before any samples were analysed, the standard mix was injected. This contained 1µM 5HTP, 1µM 5HIAA, 1µM 5HT and the internal standard 1µM N-methyl5-HT, or for dopamine analysis 1µM L-DOPA, 1µM Dopamine and the internal standard 1µM N-methyldopa. This was injected at regular intervals throughout the day of analysis to check the consistency of retention time profiles for each compound.

Once all samples were analysed, the peak heights were measured and compared to the standards of the day. Concentrations of each compound were calculated by comparison of the ratios of peak heights obtained in the test samples with corresponding values obtained with the standard mixture.

2.4.5 Preparation of mobile phase.

Mobile phase was made up to 2 litres and contained the following: 83% distilled water, 17% methanol, 31.2 g of 30 mM NaH_2PO_4, 0.744g of 1mM EDTA and 0.8g of 2.4 mM octane sulphonic acid (previously dissolved in 8mls of acetic acid 0.5M) for 5-HT analysis. For dopamine analysis the mobile phase was made up to 2 litres of double distilled water and contained : 2.1mls acetic acid, 11.5g citric acid monohydrate, 13.6g sodium acetate trihydrate, 2.4g sodium hydroxide, 0.2g octane sulphonic acid and 0.2g disodium EDTA

The flow rate was 0.7 ml/min for 5-HT and 0.8ml/min for dopamine and the electrochemical detection set at +0.6V.

2.4.6. HPLC for plasma samples.

HPLC was used to measure 5HTP, 5HT, and 5HIAA in plasma samples. No dopamine was measured in plasma as this was undetectable. The method was essentially as described above. The mobile phase was made up to 2 litres and

contained the following : 95% distilled water, 5% MeOH, 31.2g of 0.1M NaH_2PO_4, 0.744g 1 mM EDTA and 0.5g of 2.4 mM octane sulphonic acid.

2.4.7. Aldosterone radioimmunoassay.

As in section 2.2.4.

2.4.8. Corticosterone Radioimmunoassay.

Corticosterone was measured in the medium from cell stimulations by RIA (Al-Dujali & Edwards, 1981). Standards for corticosterone were prepared from solid corticosterone, (purchased from Sigma), and dissolved in ethanol and diluted in phosphate buffer (0.5% BSA). Standards were in the range 0.5-200nmol/l (equivalent to 0.2-64ng/ml).

50µl of a standard, for the standard curve, or 50µl aliqout from the cell incubation were incubated in triplicate with 200µl of phosphate buffer containing ^{125}I-iodohistamine-3-mono-oxime-corticosterone (^{125}I-corticosterone) (3000-4000 cpm) and antisera (R1B4) to corticosterone to give an initial titre of 1:10 000.

The tubes were incubated overnight at 4°C. Separation of the bound and free radiolabel in each tube was carried out at 4°C by the addition of 0.5ml of dextran coated charcoal followed by centrifugation at 4000rpm for 20 minutes at 4°C. The supernatant was aspirated and the charcoal pellet, containing the free fraction, counted in an LKB multiwell gamma counter.

The lower limit of detection for the assay was 1.25 nM, and the working range for corticosterone measurement was 1.25 to 160nM. The intra and inter assay co-efficients of variation were 8 % and 14% respectively.

2.4.9. Plasma aldosterone radioimmunoassay.

Plasma aldosterone was also measured by RIA as described above. Historically this RIA uses neat charcoal stripped plasma as a diluent for the standards etc.. The preparation of this solution is laborious and necessitates a large animal cull. It was decided to compare it with a solution containing 4% BSA, which would act as a replacement for the neat charcoal stripped plasma. Standard curves were set up and compared via analysis of variance (n=6), and no statistical significant difference between the two standard curves was observed. Thus, 50 µl of 4% BSA or 50 µl of standard containing a known concentration of aldosterone for the standard curve were incubated in triplicate with 100 µl of phosphate citrate buffer containing ^{125}I-aldosterone (approximately 4000 cpm) and 100 µl antisera (402L) to aldosterone diluted to give a final titre of 1:40 K. The tubes were incubated overnight at 4°C and treated as described in section 2.2.4.

The lower limit of the assay was 0.195nM and the working range from 0.195nM to 25nM. The intra and inter assay co-efficients of variation were 7 % and 15% respectively.

2.4.10. Plasma Renin Activity Radioimmunoassay.

Plasma renin activity (PRA) was evaluated by measuring Angiotensin I (AI) generated from sheep substrate at 4°C and 37°C (Haber *et al.*, 1969). The preparation of I^{125}-iodinated AI for RIA will be described in section 2.4.11. 10µl of plasma was mixed with 190µl of a 'premix' containing 400 parts sheep substrate, 415 parts phosphate buffer, and 20 parts each of EDTA, British anti-lewisite (BAL) and 8-OH-quinoline. The mixture, prepared on an ice bath, was then divided and one half incubated at 37°C for 4 hours, whilst the remaining half was maintained at 4°C, to measure endogenous AI. AI was measured using an RIA. 25µl of each of the plasma samples or 25µl of a known concentration of AI for the standard curve were incubated in triplicate with 200µl of phosphate buffer containing ^{125}-AI (3000-

4000 cpm) and antisera to AI (R5B4) diluted to give a final titre of 1:10,000. Standards were made up in the premix solution. The assay was set up on ice.

The tubes were allowed to equilibrate overnight at 4°C. Free and bound radiolabel were separated using dextran-coated charcoal as previously described. For this assay the results are expressed in mass rather than molar terms, this is in accordance with the expression of PRA data in many journals. The lower limit of detection for this assay was 0.5ng/ml and the working range was from 0.5 to 64ng/ml. The PRA was calculated by subtracting the concentration of AI in the sample maintained at 4°C from that of the sample incubated at 37°C. The results were corrected for the initial 20 fold dilution and the 4 hour incubation period. PRA was expressed in ng AI/ml/hour. The intra and inter assay co-efficients of variation were 8% and 16% respectively.

2.4.11. Iodination of Angiotensin I.

10µl of 10mg/ml AI was mixed with 10µl 0.5M phosphate buffer, pH 7.4 in a polypropylene tube. To this 10µl $Na^{125}I$ was added, and the solution was vortexed. 10µl of chloramine T (10mg diluted in 10ml 0.5M phosphate buffer pH 7.4), was then added and the solution vortexed and then incubated for 60 seconds. To stop the iodination reaction, 10µl sodium metabisulphite (20mg diluted in 10ml 0.5M phosphate buffer pH 7.4) was added and vortexed before the addition of 500 µl 0.1M phosphate buffer (pH 7.4), and vortexing the contents a final time.

The ^{125}I-AI was then purified by gel chromatography through a 60cm column of DEAE Sephadex A25, equilibrated with 0.1M phosphate buffer pH 7.4. The iodination mixture was applied to the column, allowed to flow through it and rinsed through with the further addition of 1ml phosphate buffer. Buffer was then pumped through the column at a flow rate of 0.25ml/minute. Fractions were collected every 4 minutes into numbered polypropylene vials. 10µl aliquots from these fractions

were counted for 60 seconds in a gamma counter to locate the ^{125}I peaks. The first to appear was the unreacted iodine and was discarded. Second to appear was the iodinated AI, the highest fraction plus the two highest on each side were collected and pooled, all other fractions were discarded. The pooled fractions were made up to 50ml with 0.1M phosphate buffer pH 7.4, containing 0.1% (w/v) sodium azide and 0.5ml Trasylol (200 kallikrein inhibitor units/ml). 1ml aliquots were placed in polypropylene tubes (LP4), capped and stored at –20°C.

2.4.12. Protein assay.

1ml of NaOH was added to the tissue retained from the incubation experiment described under 'salt diets'. The microtube was vortexed and sonicated by applying 3 short bursts that lasted approximately 3 seconds. Sonication was repeated for all the samples to ensure that all the tissue was dissolved. The tubes were then centrifuged and protein concentration was measured by a centifugal analyser, using the technique described by Bradford (1976), Dept. of Clinical Biochemistry, Royal Infirmary, Edinburgh.

2.4.13. Immunohistochemistry.

Rats on a normal salt diet were killed by decapitation. The adrenal/kidney was dissected and trimmed of all fat, fixed in 10% neutral formalin for 24 hours then embedded in paraffin. 4μM transverse sections of the adrenal/kidney were cut on a cryostat and transferred to a gelatine-coated slide. Immunostaining was by a streptavidin-biotin complex peroxidase method preceded by antigen retrieval using microwave according to the method described by Gerdes et al, (1992). Negative controls consisted of replacement of the primary antibody with an equivalent concentration of normal rabbit serum.

The sections were rinsed in water via xylene and alcohol, rinsed and wiped dry. They were then immersed in 500mls of citrate buffer at pH 6 and microwave

treatment applied for 20 minutes. Endogenous peroxidase was blocked by immersion in 3% aqueous H_2O_2 for 5 minutes. The sections were then rinsed in tap water for 3-5 minutes, then wiped dry. Before primary antibody was added the sections were incubated with 20% normal sheep serum to reduce background staining. This was drained off and primary antibody added. Incubations in the primary L-AAAD antibody were 30 minutes at room temperature and the dilution established via a titration series resulting in a dilution of 1:2000. Following a 10 minute wash in TBS, sections were incubated in biotin-conjugated anti-rabbit link antibody (diluted 1:400) for 30 minutes. A further 10 minute wash in TBS preceded incubation in StreptABC-peroxidase reagent (Dako Ltd.) for 30 minutes. After another 10 minute TBS wash peroxidase was visualised by a standard hydrogen peroxide/diaminobenzidine substrate/chromagen solution. Finally, sections were counterstained in haemotoxylin, dehydrated in ethanol, cleared in xylene and mounted in DPX.

2.5. The 5-HT transporter Study.

2.5.1. Isolated ZG cell incubations.

Isolated rat ZG cells were prepared as previously described. 100µl of the cell suspension, diluted to contain ~20,000 zona glomerulosa cells, was added to eppendorf tubes containing 100µl of Medium 199 supplemented with 5g/L BSA;2g/L D-glucose, in which all the test substances (10^{-10}M-10^{-4}M) had been dissolved to the required final concentration. Dose response curves to 5-HT, DMI, citalopram and MDMA (10^{-10}M-10^{-4}M) were obtained. These incubations acted as "controls" to allow comparison of the incubations containing 5-HT plus either DMI, citalopram or MDMA. Incubations with 10^{-10}M-10^{-4}M 5-HT were then incubated in the presence of 10^{-9}M or 10^{-6}M DMI or citalopram, or 1µM or 10µM MDMA. Control incubations contained zona glomerulosa cells and Medium 199 only, allowing the basal value for aldosterone secretion into the medium to be measured. Each incubation was carried out in triplicate and the dose response to 5-

HT carried out at least twice during each individual experiment to allow comparison between values for 5-HT and other compounds. The tubes were mixed thoroughly and incubated for 1 hour at 37°C in a shaking water bath. After 1 hour the tubes were placed on ice to prevent further steroid production and then centrifuged at 15,000g for 2 minutes at 4°C to pellet the cell suspension. The supernatant was decanted and the samples stored at –20°C until measurement of aldosterone secretion into the medium via direct radioimmunoassay (RIA).

2.5.2. Superfusion of Whole Rat Adrenal Capsules or Isolated ZG Cells.

The capsule and adhering zona glomerulosa were removed from rat adrenal glands according to the method of Haning *et al.*, (1970). The capsules or a preparation of isolated ZG cells (prepared as discussed previously) were then placed in a suspension of Sephadex in saline (0.2mg in 3mls saline), on top of nylon gauze, and superfused, at a rate of 1ml/1min, with Krebs-Ringer bicarbonate buffer (0.2% BSA) for 1 hour at 37°C to allow the tissue to equlibrate. Before addition of 5-HT and/or DMI, citalopram or MDMA, samples were collected to allow measurement of basal aldosterone levels. 5-HT (10^{-9}M-10^{-5}M) either in the presence or absence of 10^{-9}M or 10^{-6}M DMI, 10^{-9}M or 10^{-6}M citalopram or 1μM or 10 μM MDMA was added to the perfusing medium for 10 minutes and after addition of each concentration of 5-HT a wash-out period of 30 minutes followed. Samples of perfusate were collected every 5 minutes. The samples were measured for aldosterone by direct RIA.

2.5.3. *In Vivo* study of the effects of MDMA in Rats.

Female Wistar rats (200-250g;n=6) were anaesthetized with halothane in a mixture of oxygen (30%) and nitrous oxide (70%) and cannulae inserted into both femoral arteries (for the measurement of arterial blood pressure and the sampling of arterial blood), and both femoral veins (for the injection of MDMA or saline). All surgery was performed by fully trained and experienced personnel and complied with local

ethical codes and UK government regulations. A loose fitting plaster cast was applied around the pelvis and lower abdomen and pelvis with care taken to ensure that respiratory movement of the thorax was not compromised. The rats, thus restrained and supported on blocks, were allowed to recover for at least 2 hours before the study commenced. Arterial blood pressure and rectal temperature were recorded throughout the study. Blood samples were taken before the administration of MDMA (Saline for control animals). MDMA was dissolved in saline (5mg/ml) and rats were injected i.v. with 5mg/kg made up to a final volume of 0.6ml with excess saline (n=6) or with the same volume of saline alone (n=6). Once the MDMA was administered blood samples (approx. 250µl), were taken every minute for 10 minutes, and again at 20 and 30 minutes. At 30 minutes the animals were sacrificed and the adrenal glands removed. Blood was treated with ice cold EDTA (27mmol/l), to enable measurement of aldosterone by direct radioimmunoassay (RIA) and Plasma Renin Activity (PRA) by RIA.

2.5.4. [^{3}H] Paroxetine Binding.

Sections of whole rat adrenal gland were cut in a cryostat (-20^{o}C), thaw-mounted onto gelatin covered glass slides and stored at -70^{o}C for subsequent [^{3}H]paroxetine autoradiography binding analysis of 5-HTergic uptake sites. The slide mounted sections were processed for [^{3}H]paroxetine autoradiography according to the protocol described by Battaglia *et al.*, (1987). Briefly, sections were incubated in Tris buffer containing a saturating concentration (250pM) of [^{3}H]paroxetine. Non-specific binding was defined in adjacent sections by [^{3}H]paroxetine binding in the presence of 4µM citalopram. Following incubation, the sections were washed in buffer, dipped in deionized water and rapidly dried under a stream of cold air. The sections, together with a series of [^{3}H] standards were apposed to X-ray film in a light tight container and stored at -70^{o}C for 4-6 weeks.

Analysis of [^{3}H]paroxetine autoradiographs was performed using a computer based image analysis system (Cambridge Instruments Quantimet 970). Tissue isotope concentrations were measured from autoradiographic images of brain sections, relative to appropriate [^{3}H] containing standards (Amersham International). Specific [^{3}H]paroxetine binding was determined by subtraction of image densities of non-specific binding images from total binding images.

2.5.5. Western Blotting.

Cell pellets of adrenal capsular tissue and adrenal medulla/fasciculata tissue were prepared by adding the tissue to 5 parts of PBS, and homogenising in a glass/glass hand held homogeniser. The homogenate was then centrifuged at 15,000g for 10 minutes at 4^{o}C, and the supernatant decanted. The cell pellet was then washed with PBS, and centrifuged as above. The supernatant was then removed and the cell pellet stored at -70^{o}C prior to Western blotting.

The cell pellet was defrosted and PBS was added (enough to dilute the protein in each sample to ~50-100µg per lane). Once diluted the cell pellet was vortexed and an equivalent amount of Laemmli added and vortexed. For each capsule 400µl PBS and 400µl Laemmli was added and for medulla/fasciculata tissue 800µl PBS and 800 µl Laemmli. Once vortexed the cell pellets are incubated at 37^{o}C for 30 minutes. 50µl of Laemmli was then added to 50µl of 'rainbow markers', and this was incubated at 100^{o}C for 10 minutes.

The samples were then added to a gel (50µl of each sample per lane), and electophoresis was carried out at 25-30mA, for 4 hours. The gel was then transferred overnight at 4^{o}C onto a membrane. To prepare a gel, 2 glass plates were rinsed in water and ethanol and swabbed with acetic anhydride, and allowed to dry. The plates were then clamped onto the appropriate stand. Resolving gel was added, slowly down one side of the plate, leaving ~3cm at the top of the plate

for addition of stacking gel. A thin layer of water saturated butan-1-ol was then added to avoid the formation of a meniscus. Stacking gel was added and a comb inserted immediately. This was allowed to set. The comb was then removed, the gel rinsed and filled with reservoir buffer. To transfer a gel, a sponge and blot paper was added to a cassette , soaked in transfer buffer, and the membrane placed on top of the blot paper. The gel was added onto the membrane, then another blot paper and then finally a sponge. The cassette was then shut over ready for transfer overnight.

The membrane was then blocked with 5% Marvel and 0.05% Tween 20 in TBS for 1 hour. The membrane was then incubated with transporter antibody at a 1:200 dilution for 1 hour, washed with 2% Marvel, 0.05% Tween 20 in TBS, then incubated with horseradish peroxidase conjugated secondary antibody (1:1000) for 1 hour. Bands were detected by ECL with DAB and photographic film.

2.5.6. Immunohistochemistry.

Rats on normal salt diet were killed by decapitation. The adrenal was dissected and trimmed of all fat, fixed in 10% neutral formalin for 24 hours then embedded in paraffin. 4µm transverse sections of the adrenal/kidney were cut on a cryostat and transferred to a gelatin coated slide. Immunostaining was by an indirect method of antigen retrieval using microwave according to the method described by Gerdes *et al*, (1992). Negative controls consisted of replacement of the primary antibody with an equivalent concentration of normal rabbit serum.

Two antibodies to the 5-HT transporter were studied. The first, S (5-HT)-240-KLH (keyhole lympet haemocyanon), was raised in rabbits against a 14 amino acid sequence present in the second extracellular loop of the 5-HT transporter, a sequence chosen as it is similar to that used for the dopamine transporter antibody, which had proven very successful (Quian *et al.*, 1995). The second antibody used,

S-387-KLH, was again raised in rabbits, but this time against a 14 amino acid sequence contained in the fourth extracellular loop, a peptide sequence that corresponds to that used by Blakely *et al.*, (1992), for generation of his 5-HT transporter antibody

The sections were rinsed in water via xylene and alcohol, rinsed and wiped dry. They were then immersed in 500mls of citrate buffer at pH 6 and microwave treatment applied for 20 minutes. Endogenous peroxidase was blocked by immersion in 3% aqueous H_2O_2 for 5 minutes. The sections were then rinsed in tap water for 3-5 minutes, then wiped dry. Before the primary antibody was added the sections were incubated with 20% normal sheep serum to reduce background staining. This was drained off and primary antibody added. Incubations in the primary antibody were 60 minutes at room temperature and the dilution established via a titration series resulting in a dilution of 1:200 for both antibodies. Following a 10 minute wash in TBS, sections were incubated in peroxidase conjugated anti-rabbit link antibody (diluted 1:50) for 60 minutes. A further 10 minute wash in TBS preceded incubation in StreptABC-peroxidase reagent (Dako Ltd.) for 30 minutes. After another 10 minute TBS wash peroxidase was visualised by a standard hydrogen peroxide/Diaminobenzidine substrate/chromagen solution. Finally, sections were counterstained in haemotoxylin, dehydrated in ethanol, cleared in xylene and mounted in DPX.

Source of Materials.

2.6.1. Indoleamine Study.

Indole Compounds	Sigma Chemical Company Ltd. Poole, England.
Medium 199	Flow Laboratories, Irvine, Scotland.
BSA	Miles Laboratories, Slough England.
Collagenase	Worthington Biochemical Corp, USA.
Gauze	Henry Simon Ltd, Stockport, England.

Salts for Buffer Solutions	BDH Ltd, Poole, England.
Glucose	BDH Ltd, Poole, England.

2.6.2. Agonist\Antagonist Study.

5-HT	Sigma Chemical Company Ltd, Poole, England.
5-Methoxytryptamine	Sigma Chemical Company Ltd, Poole, England.
Ketanserin	Janssen Pharmaceuticals, Wantage, England.
Methysergide	Sandoz Pharmaceuticals, Middlesex, England.
Mesulergine	Sandoz Pharmaceuticals, Middlesex, England.
Cyanopindolol	Sandoz Pharmaceuticals, Middlesex, England.
ICS 205/930	Sandoz Pharmaceuticals, Middlesex, England.
Zacopride (R)	Synthelabo Recherche, France.
Zacopride (S)	Synthelabo Recherche, France
Cisapride	Synthelabo Recherche, France.
BIMU 1	Boehringer Ingelheim, Italy.
BIMU 8	Boehringer Ingelheim, Italy.
DAU 6285	Boehringer Ingelheim, Italy.

2.6.3. L-AAAD Study.

5-HT, 5-HTP, 5-HIAA,	Sigma Chemical Company, Poole, England.
Dopamine, L-DOPA,	
N-methyl5-HT, N-methyldopa.	
L-Cysteine	Sigma Chemical Ltd, Poole, England.
Pargyline	Sigma Chemical Ltd, Poole, England.
Carbidopa	Merck, Sharp and Dome, West Point, USA.
Perchloric Acid	May & Baker, Manchester, England.
HPLC Grade Water	Rathburn Chemicals Ltd, Walkerburn, Scotland.
HPLC Grade Methanol	Rathburn Chemical Ltd, Walkerburn, Scotland.
Salts for Mobile Phase	BDH Ltd, Poole, England.

Thrombin	Sigma Chemical Company Ltd, Poole, England.
EDTA	BDH Ltd, Poole, England.
Clomipramine	Ciba Laboratories, Horsham, England.
StreptABC Peroxidase Reagent	Dako Ltd, England.
L-AAAD Antibody	Kindly donated by Professor Nagatsu, Japan.
Immunohistochemistry	All reagents kindly donated by L. Brett, Pathology Dept., WGH, Edinburgh.

2.6.4. Transporter Study.

Sephadex	BDH Merck, England.
DMI	Sigma Chemical Company Ltd, Poole, England.
Citalopram	Sigma Chemical Company Ltd, Poole, England.
MDMA	Sigma Chemical Company Ltd, Poole, England.
In Vivo MDMA Reagents	Clinical Neuroscience Dept., WGH, Edinburgh.
[^{3}H] paroxetine binding	Clinical Neuroscience Dept., WGH, Edinburgh.
Immunohistochemistry	Pathology Dept., WGH, Edinburgh.
Western Blotting	All reagents kindly donated by Dr. J. Lawrence, Pharmacology Dept., University of Edinburgh.

2.6.5. Miscellaneous.

Gelatin	Sigma Chemical Company Ltd, Poole, England.
Dextran T-70	Pharmacia Ltd, Milton Keynes, England.
BAL	Sigma Chemical Company Ltd, Poole, England.
8-OH-Quinoline	Sigma Chemical Company Ltd, Poole, England.
Arachis Oil	Hospital Pharmacy.

2.7. Buffers/Solutions etc.

Medium 199:-1 sachet of medium 199 was dissolved in 100ml of distilled water and a 15ml aliquot was added to 210ml of salt solution containing $NaHCO_3$, $MgSO_4$, KH_2PO_4, $CaCl_2$ and NaCl. The final concentration of ions in the medium in mM were, sodium 145, potassium 3.9, calcium 2.5, magnesium 1.2, bicarbonate 25, chloride 128, phosphate 1.2 and sulphate 1.2.

Phosphate Buffer:-This solution contained 80 mM Na_2HPO_4, 20mM $KH_2PO_4$and 15mM NaN_3 made up in distilled water (pH 7.4). For use in assays 0.1% BSA was added.

Dextran Coated Charcoal:-6.25g of charcoal, 0.4g of gelatin pre-dissolved in 20ml of warm phosphate buffer and 0.62g of dextran in 1 litre of phosphate buffer (pH 7.4).

Sodium Acetate Buffer:-120ml acetic acid (0.1M) and 80ml of sodium acetate (0.1M) (pH 4.75).

EDTA Inhibitor for Blood Collection:- 27mM EDTA in 0.9% saline.

Cocktail for Blood Collection:-1.1 μM clomipramine, 11.1μm pargyline and 1.1 units thrombin/ml, in distilled water.

Phosphate/Citrate Buffer:-20mM citric acid and 12mM NaH_2PO_4in distilled water (pH4).

EDTA for AI RIA:-270mM EDTA in distilled water.

BAL:-15ml arachis oil, 242μl 2,3-dimercapto-propan-1-ol and 535ml benzyl benzoate.

8-OH-Quinoline:-454mM 8-hydroxyquinoline in distilled water.

Krebs Ringer Buffer:-120mM NaCl, 2.6mM KCl, 1.2mM $CaCl_2$, 1.18mM KH_2PO_4, 1.18mM $MgSO_4$and 25.8mM $NaHCO_3$, made up in distilled water (pH 7.4).

Tris Buffer:-30mM Tris and 15mM NaN_3 made up in 30mM HCl (pH 7.4).

Citrate Buffer:-100mM citric acid, 300mM NaOH, 1mM EDTA and 1g/litre of gelatin, made up in distilled water (pH 6.2).

4% BSA:-4g of BSA in 100mls phosphate buffer.

Stacking Gel Buffer:-0.5M Tris HCl (pH 6.8). 6.0g Tris in 40ml distilled water, add ~48ml of 1M HCl to bring the pH to 8, and make up to 100ml with distilled water. Filter through Whatman No.1 paper, and store at 4°C.

Resolving Gel Buffer:-3.0M Tris HCl (pH 8.8). 36.3g Tris in 48ml 1M HCl, and made up to 100ml with distilled water. Filtered and stored at 4°C.

Reservoir Buffer:-0.25M Tris HCl (pH 8.3). 30.3g Tris in 48ml 1M HCl, 1.92M glycine (144.0g) and 1% SDS (10g). Made up to 1000ml with distilled water and stored at 4°C.

Stacking Gel:-40% acrylamide solution (1.8ml), 2% bisacrylamide solution (0.9ml), stacking gel buffer (5ml), 10% SDS (0.2ml), 1.5% Ammonium Persulfate (1ml), distilled water (11.1ml) and Temed (added at the last minute)(0.015ml).

Resolving Gel:-For a 7.5% solution; 40% acrylamide (5.63ml), 2% bisacrylamide (3ml), resolving gel buffer (3.75ml), 10% SDS (0.3ml), 1.5% ammonium (1.5), distilled water (15.82) and Temed (0.015ml)

Sample Buffer (Laemmli):-For a 7.5% solution; Tris HCl 0.0625M (pH 6.8). Tris HCl (0.76g), 2% SDS (20mls), 5% 2 mercaptoethanol (5ml), 1mm DTT (1ml), 10% glycerol (20ml), 0.002% bromophenol blue and water to 100mls.

Transfer/Running Buffer:-Tris HCl (25mM) and glycine (192mM), made to volume with distilled water. Running buffer also contained SDS (0.1%), and Transfer Buffer contained 20% methanol.

2.8. Data analysis.

Results are shown as mean ± standard error of the mean. n refers to the number of times the experiment was performed. The significance between different data sets were assessed by analysis of variance. The significance between basal and different concentrations of agonist/antagonist in an experiment was assessed by Student's t – test. *P value of < 0.05, **P < 0.01 and ***P < 0.001 were considered statistically significant.

Chapter Three

Indoleamine-Stimulated Aldosterone Secretion - Structure Activity Relationships.

3.1. Introduction.

Despite the fact that 5-HT is known to stimulate aldosterone secretion from the ZG, the physiological relevance of this remains poorly understood, and the role of other indoleamines, which occur naturally, has not been fully evaluated. Variations in the structure of tryptamine and 5-HT have mainly been made in a search for antagonists to 5-HT; much less work has been published, however on the relative potency of tryptamine derivatives as mimics of 5-HT. At the time that 5-HT was shown to have stimulatory activity on the ZG there was some disagreement on the assumption that 5-HT was the most important physiological indoleamine agonist. A carboline metabolite of the indoleamine melatonin was believed by some to have 100 times the potency of 5-HT in stimulating aldosterone release (Farrell, 1961). However this carboline was shown to be inactive *in vitro* (Jouan, 1963). Mendelsohn *et al.*, (1981) reported that an unidentified component of rat plasma was able to stimulate aldosterone secretion beyond the maximal stimulation by 5-HT. The fact that this stimulation was depressed by 5-HT antagonists indicated the possibility that there could exist an endogenous 5-HT receptor agonist with activity superior to that of 5-HT.

A few studies in the adrenal gland comparing a number of indoleamines have been published. Muller (1968), tested nine compounds related to 5-HT in adrenal quarters in an attempt to discover an alternative physiological agonist, the study did not, however, look at the effects of these compounds in isolated ZG cells, making interpretation of these results difficult. Al-Dujali *et al.*, (1982) using rat ZG cells in a column perfusion system demonstrated that 5-HT, 5-MeOT and to a lesser extent 5-methoxytryptophol and melatonin were able to stimulate aldosterone secretion. 5-MeOT appeared to be more potent than 5-HT in this system. In 1970, Muller was able to show a stimulatory effect of 5-HT, 5-MeOT and L-5-hydroxytryptophan in static incubates of adrenal slices. Thus despite considerable

understanding of the metabolic pathway of 5-HT, little is known of the ability of other indoleamines to cause aldosterone secretion.

In this chapter, these previous studies have been extended using a more pharmacological approach. 5-HT and a range of related indoleamines, including metabolites and precursors and synthetic congeners, were all incubated with isolated rat ZG cells. In addition to the measurement of aldosterone secretion, the second messenger for 5-HT, cAMP, was also quantitated. The rationale behind this study was two fold, firstly to determine how specific 5-HT stimulated steroidogenesis is in relation to other structure-related indoleamines and to define more clearly the structure-activity requirements for this stimulation in the ZG cells, and secondly to determine if the metabolites, precursors or intermediates of the 5-HT biosynthetic and metabolic pathways could give rise to a steroidogenic response.

3.2. Statistical Analysis.

Statistical significance was calculated in the individual experiments by ANOVA and then Student's t-test for unpaired samples. A p value of <0.05 was considered significant. NS indicates non-significance. *, ** and *** indicates that $p<0.05$, $p<0.01$ and $p<0.001$ respectively. P values relating to the mean values from the 4 separate experiments were calculated.

3.3. Results.

The graphs illustrated show mean±SEM of four individual experiments, and within a single experiment each incubation was performed in triplicate. Within each individual experiment two identical dose response curves to 5-HT were carried out, i.e. the first assay to be set up and the last. This provided the standard against which the stimulations by other indoleamines could be quantified. Although the cell number remained constant within each experiment, from experiment to experiment

small variations occurred in the basal values for aldosterone secretion into the medium. For this reason aldosterone secretion is expressed as the stimulation ratio±SEM in relation to the basal secretion of the control samples which have been given a value of 1. This normalization procedure is commonly used in studies involving dispersed cells and cells in primary culture. Variations in the responsiveness of the cells to a particular stimulus were also observed between experiments, and may relate to differing degrees of cellular damage caused by the collagenase digestion (Vinson *et al* 1985).

The cAMP response was only studied in one experiment (in order to establish that the compounds which stimulated aldosterone secretion, also stimulated cAMP). For this reason it was not considered valid to conduct statistical analysis of the cAMP data.

The mean basal aldosterone secretion in all experiments was 1.0±0.18 (mean ±SEM) nM per 20,000 cells per hour.

The dose responses to 5-HT observed corresponded to those already reported (Haning 1970, Albano 1974). Characteristically the aldosterone response had a threshold around 10^{-9}M 5-HT (Figure 3.1), below which there was no apparent response. Significant stimulation of aldosterone was observed at 10^{-8}M 5-HT ($p<0.05$) and 10^{-6}M 5-HT produced maximal stimulation. Though there appeared to be a reduction of the maximal effect at 10^{-5}M 5-HT this was not significant. The maximal stimulation of aldosterone release produced by 5-HT was generally in the order of four times the basal secretion rate (Figure 3.1.).

Of the compounds tested in the study four gave maximal stimulation of cAMP induced aldosterone secretion comparable to that seen with 5-HT, namely 5-methoxytryptamine, 5-methyltryptamine, N-methyltryptamine and tryptamine.

Figures 3.1.-3.6. represent the dose-response curves expressed as stimulation ratios for the compounds tested in the study. Figure 3.2. shows the aldosterone response to 5-methoxytryptamine, a minor metabolite of 5-HT, producing comparable stimulation of aldosterone to that seen with 5-HT, and threshold stimulation at 10^{-9} M with maximal stimulation at 10^{-6}M, 6-methoxytryptamine producing maximal stimulation at 10^{-5}M, higher concentrations produced no further increase in aldosterone stimulation and 5-HIAA the major metabolite of 5-HT having no aldosterone stimulating activity. Figure 3.3. shows the stimulation ratios for 5-methyltryptamine, producing a maximal stimulation at 10^{-7}M, and a threshold stimulation at 10^{-9}M and N-methyltryptamine producing comparable stimulation to that seen with 5-HT, with maximal stimulation again at 10^{-6}M and threshold stimulation at 10^{-9}M. Tryptamine produced comparable stimulation of aldosterone to that seen with 5-HT at 10^{-5}M, concentrations and above this produced no further increases in aldosterone secretion. Figure 3.4. represents the stimulation ratios for 5-hydroxytryptophan, the precursor to 5-HT, producing a weak maximal stimulation at 10^{-5}M, higher concentrations producing no further increases in aldosterone secretion, L-tryptophan, a precursor to 5-HT producing no aldosterone stimulating activity, melatonin, producing a small non-significant rise in aldosterone secretion at 10^{-5}M, and 5-hydroxytryptophol producing no aldosterone stimulating activity. Figure 3.5. and Figure 3.6. represent the stimulation ratios for 5-fluorotryptamine, 6-fluorotryptamine, 5,6-dihydroxytryptamine, 5,7-dihydroxytryptamine and 5-hydroxytryptophol, of which only 5-fluorotryptamine produced weak aldosterone stimulating activity at 10^{-5}M, higher concentrations producing no further increases in aldosterone secretion. The overall results for the compounds tested which stimulated aldosterone are shown in Figure 3.7. From the raw data, EC_{50} and maximal values were calculated by a curve fitting procedure (software provided by Dr. R. Barlow).

From the data gained in this study we were primarily interested in the EC_{50} values for the compounds which displayed aldosterone stimulating activity, that is the concentration required for each agonist to produce half maximal response, and the % maximal stimulation for each compound compared to the maximal stimulation produced by 5-HT. This would then enable comparison of each compound with 5-HT and thus calculation of the important structural requirements for aldosterone stimulating activity. This data is summarised in Table 3.1.

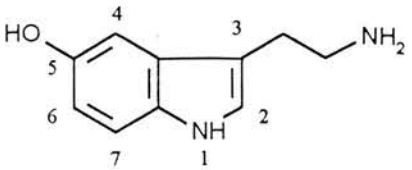
The EC_{50} and the % maximal stimulation values are the mean±SEM from 4 individual experiments. The indole nucleus at the top of Table 3.1. shows the groupings R_1 and R_2 of which we were interested in looking at the differing groups.

5-HT, 5-methyltryptamine and 5-methoxytryptamine all contain an amine grouping at R_1 and an hydroxy, methyl and methoxy group respectively at R_2, and all have similar low EC_{50} values and produce comparable maximal stimulations of aldosterone. N-methyltryptamine containing an N-methyl group at R_1 and no R_2 group also has a low EC_{50} value similar to that of 5-HT and produces comparable maximal stimulation of aldosterone. However tryptamine containing an amine group at R_1 and again no R_2 group shows high maximal stimulation of aldosterone but with a high EC_{50} value. 6-methoxytryptamine with an amine group at R_1 and now with a methoxy group at C_6 has a high EC_{50} value and produces weak maximal stimulation of aldosterone. 5-HTP the precursor to 5-HT containing an amine carboxylic acid group at R_1 and a hydroxyl group at R_2 produces weak stimulation of aldosterone and has a high EC_{50} value. In contrast however , L-tryptophan also a 5-HT precursor containing an amine carboxylic acid group at R_1 but no R_2 group produces no aldosterone stimulating activity. The major metabolite of 5-HT, 5-HIAA, containing a carboxylic acid group at R_1 and a hydroxy group at R_2 also has no aldosterone stimulating activity. Melatonin,

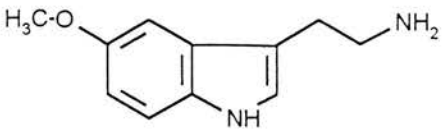
Graphical Illustration

- Chapter Three -

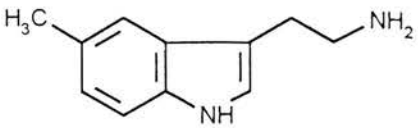
STRUCTURES OF INDOLEAMINES



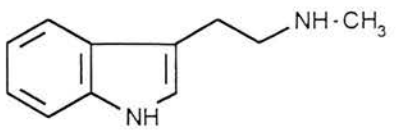
5-Hydroxytryptamine (5-HT)



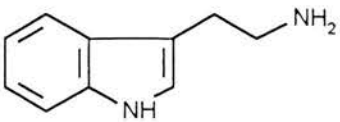
5-Methoxytryptamine



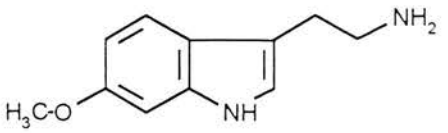
5-Methyltryptamine



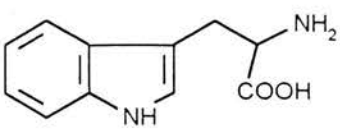
N-Methyltryptamine



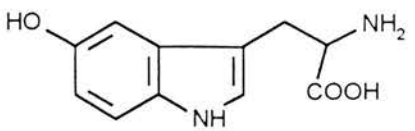
Tryptamine



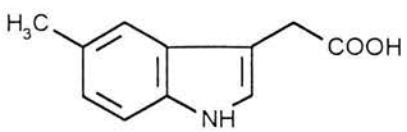
6-Methoxytryptamine



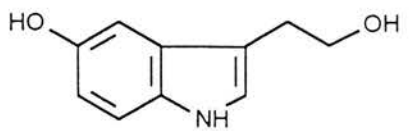
L-Tryptophan



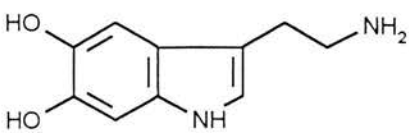
5-Hydroxytryptophan



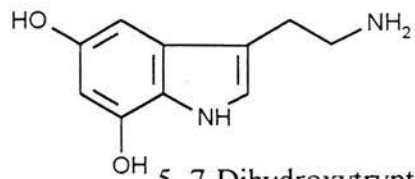
5-Hydroxyindoleacetic acid (5-HIAA)



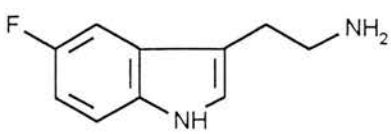
5-Hydroxytryptophol



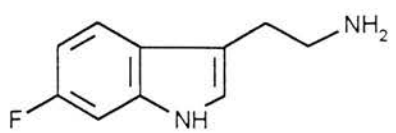
5, 6-Dihydroxytryptamine



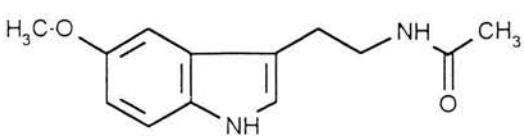
5, 7-Dihydroxytryptamine



5-Fluorotryptamine



6-Fluorotryptamine



Melatonin

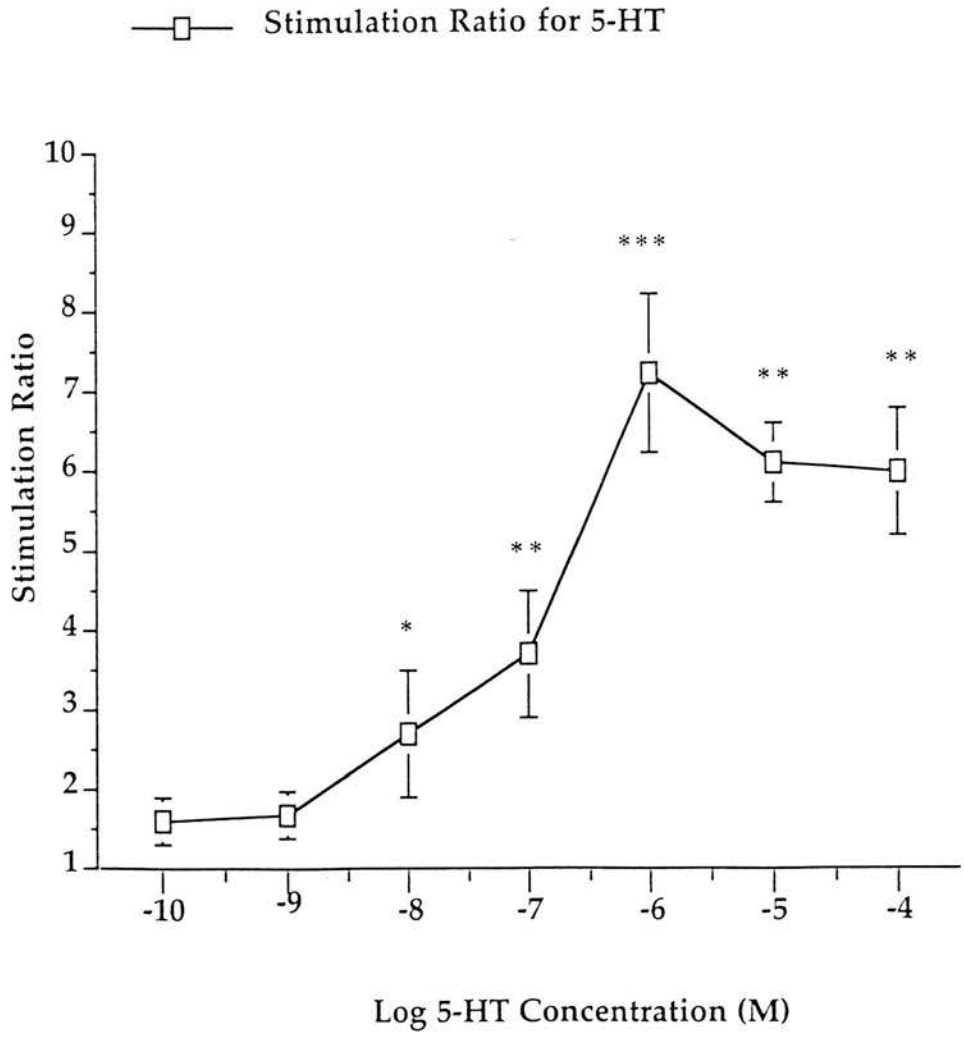


Figure 3.1. *Dose-dependent increase in aldosterone secretion from isolated rat ZG cells in response to increasing concentrations of 5-HT.* Isolated rat ZG cells were incubated for 1 hour at 37°C with increasing concentrations of 5-HT. Aldosterone secretion into the medium was measured by RIA. Data, represented as stimulation ratios, from 4 separate experiments, with triplicate incubations performed in each, were combined and expressed as mean±SEM. *$p<0.05$; **$p<0.01$ and ***$p<0.001$ compared to basal aldosterone levels.

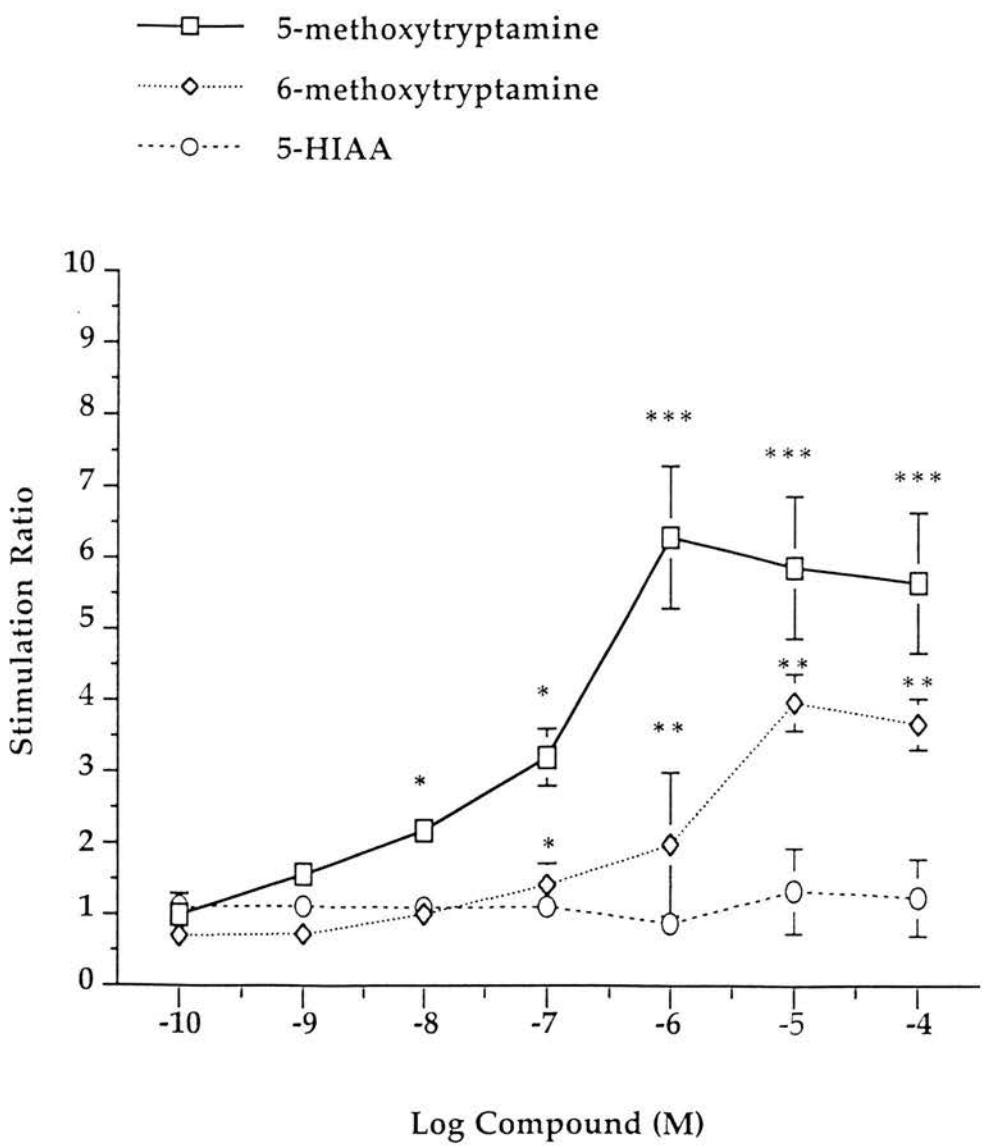


Figure 3.2. *Dose-dependent increase in aldosterone secretion from isolated rat ZG cells in response to increasing concentrations of 5-MeOT, 6-MeOT and 5-HIAA.* Isolated rat ZG cells were incubated for 1 hour at 37°C with increasing concentrations of 5-MeOT, 6-MeOT and 5-HIAA. Aldosterone secretion into the medium was measured by RIA. Data, represented as stimulation ratios, from 4 separate experiments, with triplicate incubations performed in each, were combined and expressed as mean±SEM. *$p<0.05$; **$p<0.01$ and ***$p<0.001$ compared to basal aldosterone levels.

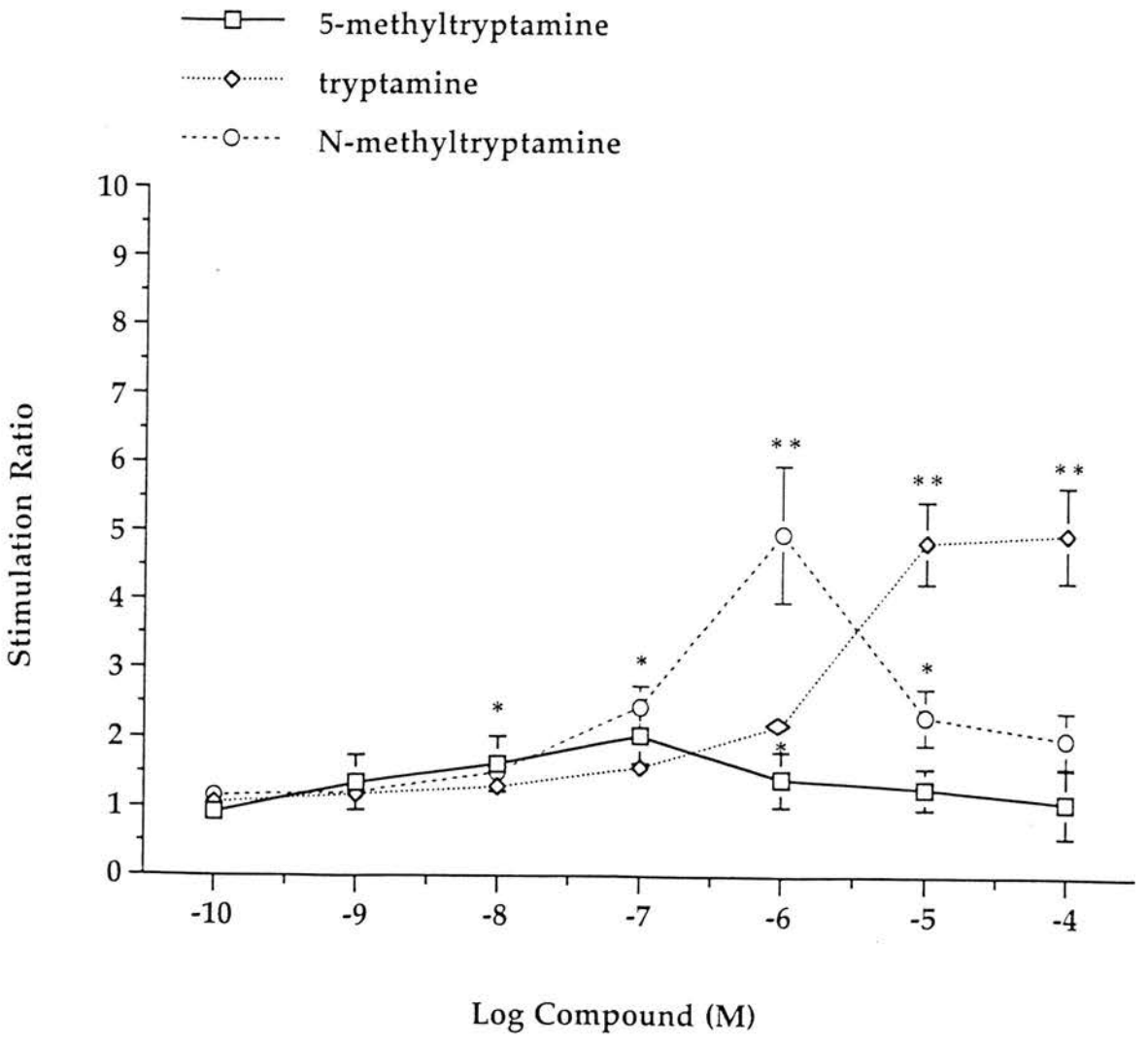


Figure 3.3. *Dose-dependent increase in aldosterone secretion from isolated rat ZG cells in response to increasing concentrations of 5-methyltryptamine, N-methyltryptamine and tryptamine.* Isolated rat ZG cells were incubated for 1 hour at 37°C with increasing concentrations of 5-methyltryptamine, N-methyltryptamine and tryptamine. Aldosterone secretion into the medium was measured by RIA. Data, represented as stimulation ratios, from 4 separate experiments, with triplicate incubations performed in each, were combined and expressed as mean±SEM. *$p<0.05$; **$p<0.01$ and ***$p<0.001$ compared to basal aldosterone levels.

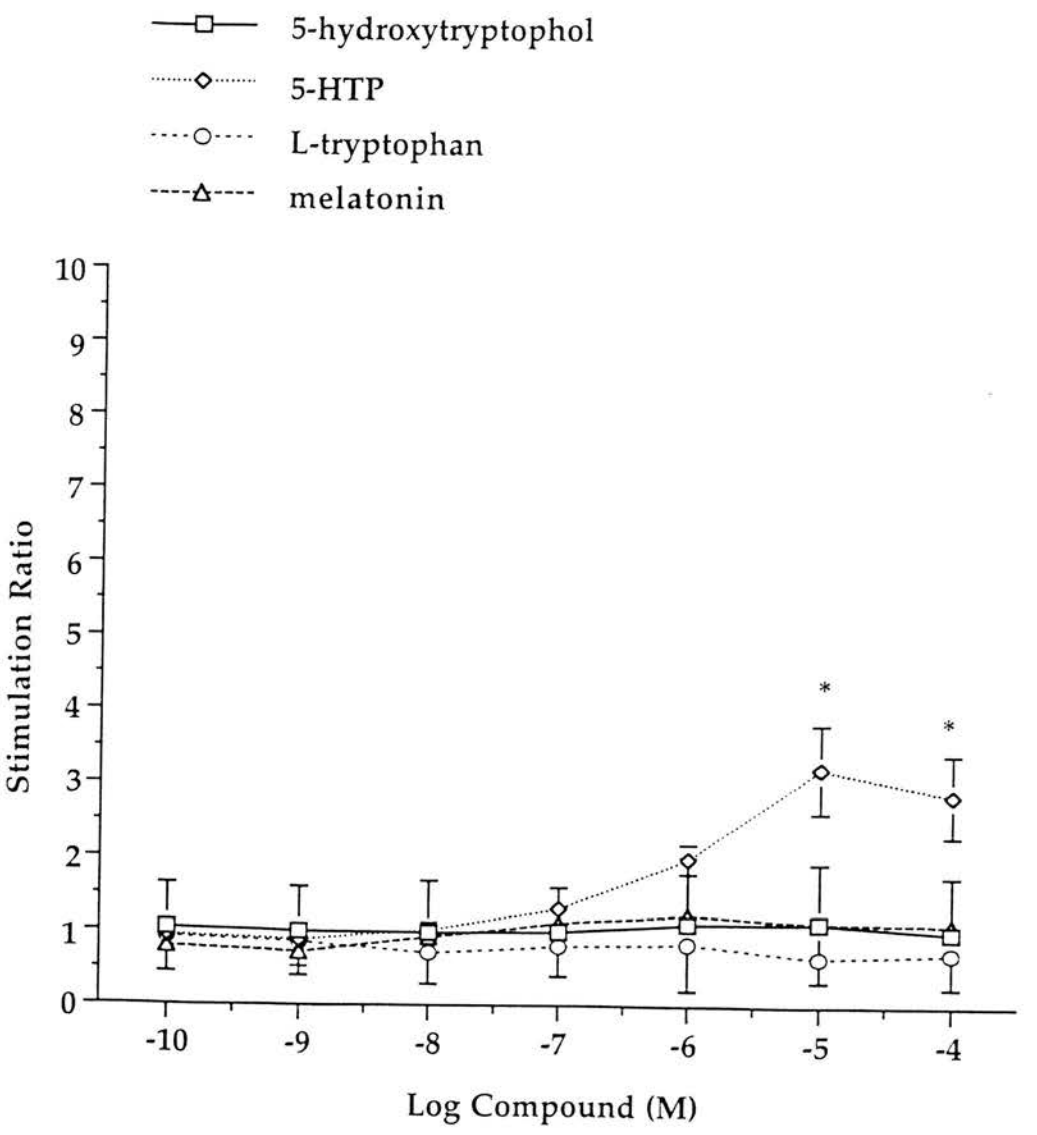


Figure 3.4. *Dose-dependent increase in aldosterone secretion from isolated rat ZG cells in response to increasing concentrations of 5-hydroxytryptophol, 5-HTP, L-tryptophan and melatonin.* Isolated rat ZG cells were incubated for 1 hour at 37°C with increasing concentrations of 5-hydroxytryptophol, 5-HTP, L-tryptophan and melatonin. Aldosterone secretion into the medium was measured by RIA. Data, represented as stimulation ratios, from 4 separate experiments, with triplicate incubations performed in each, were combined and expressed as mean±SEM. *$p<0.05$ compared to basal aldosterone levels.

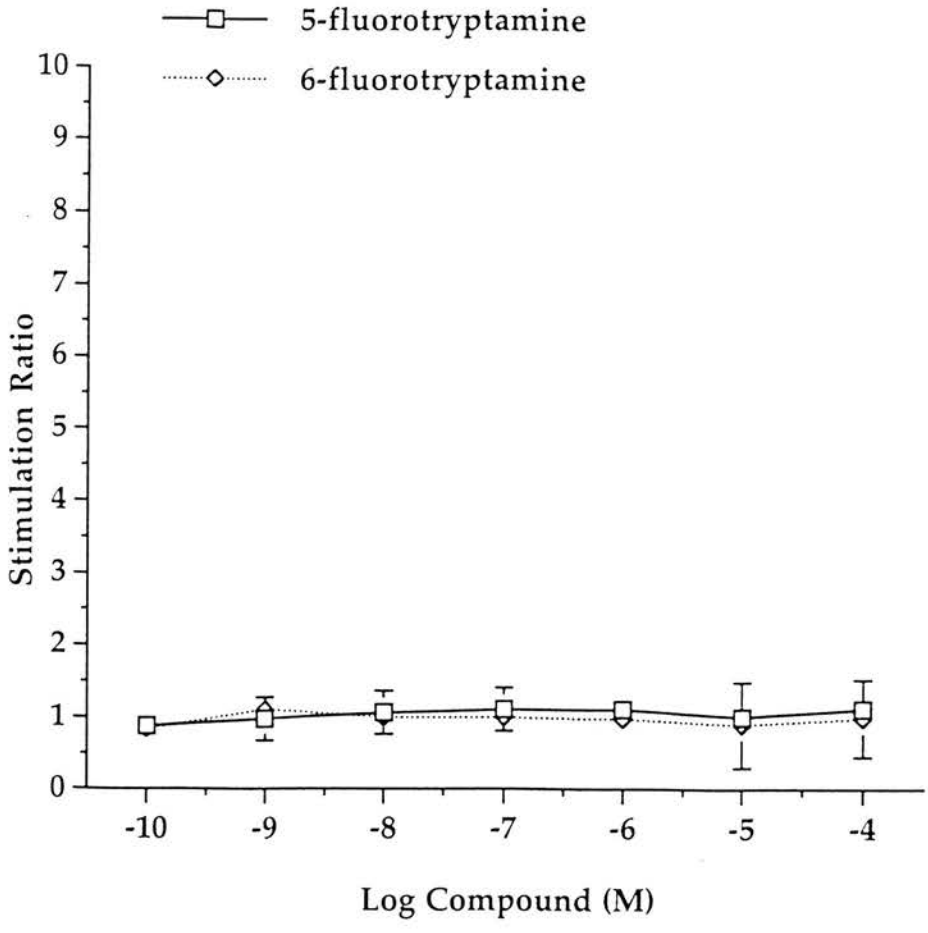


Figure 3.5. *Dose-dependent increase in aldosterone secretion from isolated rat ZG cells in response to increasing concentrations of 5-fluorotryptamine and 6-fluorotryptamine.* Isolated rat ZG cells were incubated for 1 hour at 37°C with increasing concentrations of 5-fluorotryptamine and 6-fluorotryptamine. Aldosterone secretion into the medium was measured by RIA. Data, represented as stimulation ratios, from 4 separate experiments, with triplicate incubations performed in each, were combined and expressed as mean±SEM.

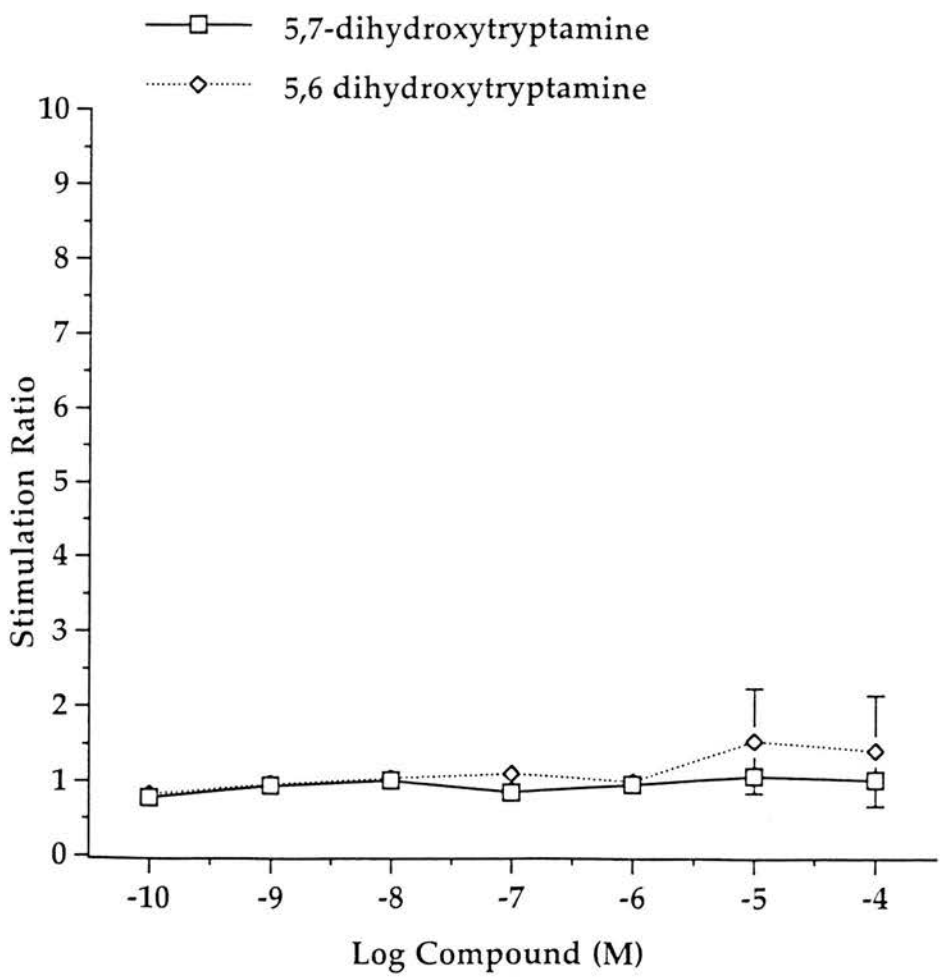


Figure 3.6. *Dose-dependent increase in aldosterone secretion from isolated rat ZG cells in response to increasing concentrations of 5,6-dihydroxytryptamine and 5,7-dihydroxytryptamine.* Isolated rat ZG cells were incubated for 1 hour at 37°C with increasing concentrations of 5,6-dihydroxytryptamine and 5,7-dihydroxytryptamine. Aldosterone secretion into the medium was measured by RIA. Data, represented as stimulation ratios, from 4 separate experiments, with triplicate incubations performed in each, were combined and expressed as mean±SEM.

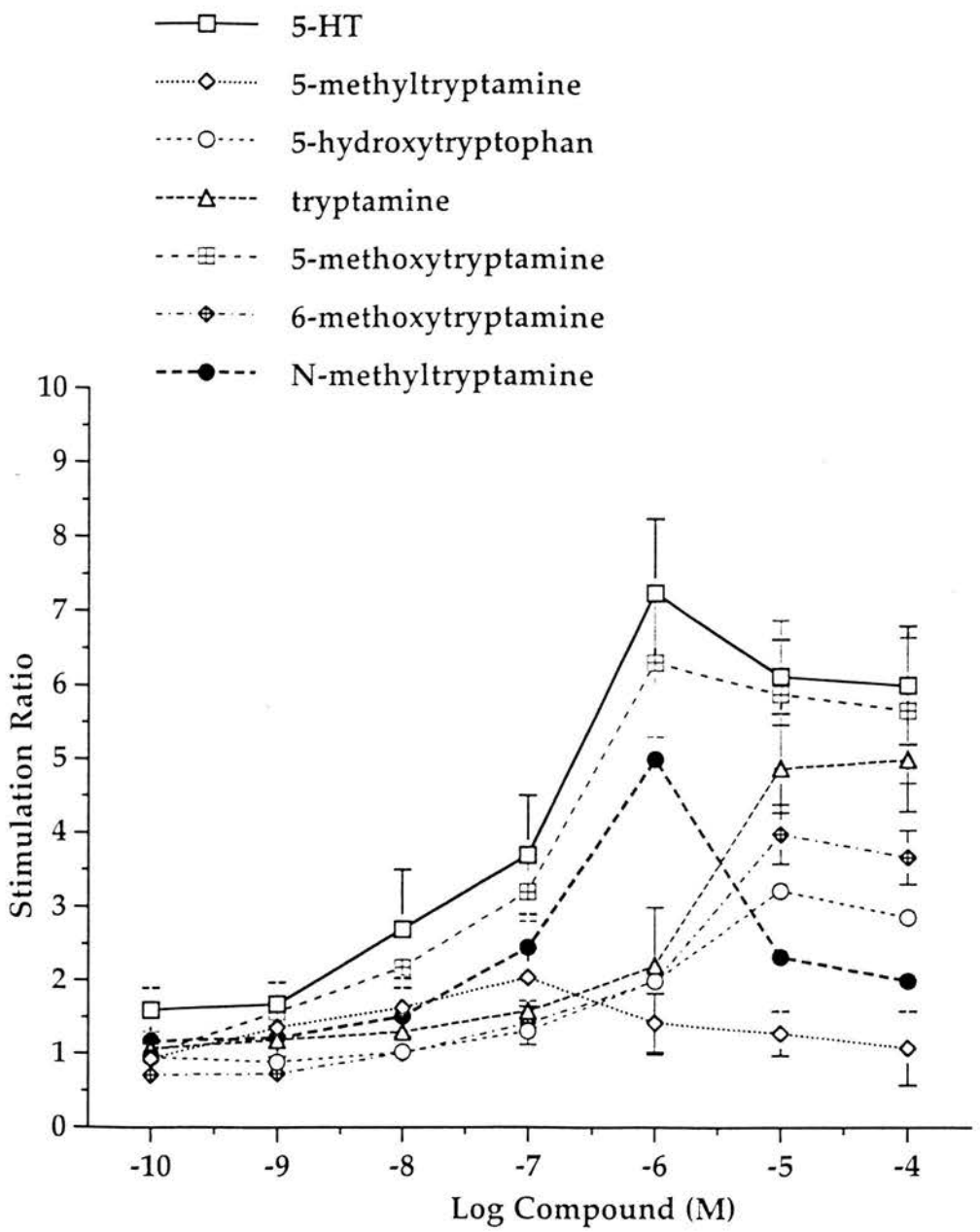


Figure 3.7. *Summary graph of the indoleamines studied which produced an increase in aldosterone secretion from isolated ZG cells.*

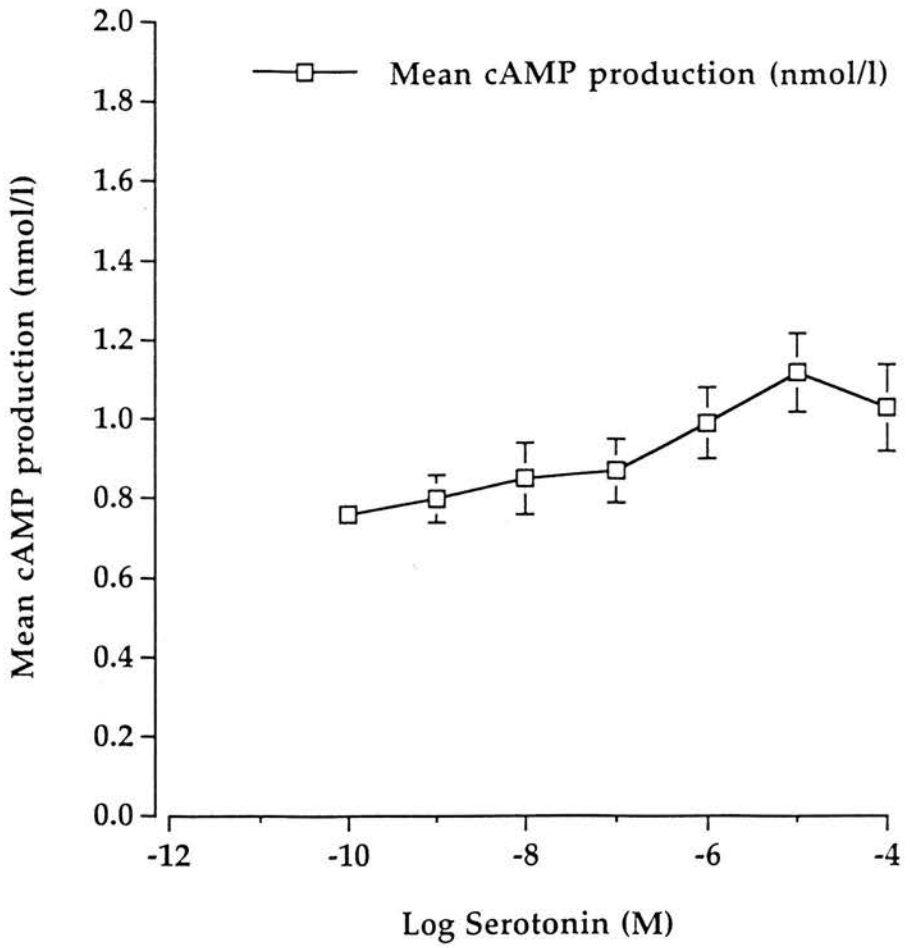


Figure 3.8. *cAMP secretion into the medium after incubation of isolated ZG cells with increasing concentrations of 5-HT.* cAMP measurements into the medium after incubating isolated ZG cells with increasing concentrations of 5-HT. The graph is representative of one experiment, with each incubation performed in triplicate.

Indoleamine	R_1 and R_2 Grouping	R_3 and R_4 Grouping	EC_{50}±SEM (μM)	Maximal Stimulation (mean±SEM)
5-HT	-HO/-NH_2	---------- -	0.145±0.027	100%
5-methoxytryptamine	-H_3CO/ -NH_2	---------- -	0.266±0.018	82%±6%
5-methyltryptamine	-H_3C/ - NH_2	---------- -	0.139±0.038	74%±12%
N-methyltryptamine	--/- $NHCH_3$	---------- -	0.127±0.023	100%±5%
Tryptamine	--/-NH_2	---------- -	2.21±0.352	140%±28%
6-methoxytryptamine	--/NH_2	-H_3CO/--	3.01±0.069	60%±4%
5-hydroxytryptophan	-HO/- NH_2COOH	---------- -	1.93±0.273	32%±9%
Melatonin	-H_3CO/- $NHCH_3O$	---------- -	---------- --	----------
L-tryptophan	--/ - NH_2COOH	---------- -	---------- --	----------
5-hydroxyindoleacetic acid	-HO/- COOH	---------- -	---------- --	----------
5-hydroxytryptophol	-HO/-HO	---------- -	---------- --	----------
5-fluorotryptamine	-Fl/-NH_2	---------- -	---------- --	----------
6-fluorotryptamine	--/-NH_2	-Fl/--	---------- --	----------
5,6-dihydroxytryptamine	-HO/-NH_2	-HO/--	---------- --	----------
5,7-dihydroxytryptamine	-HO/-NH_2	--/-HO	---------- --	----------

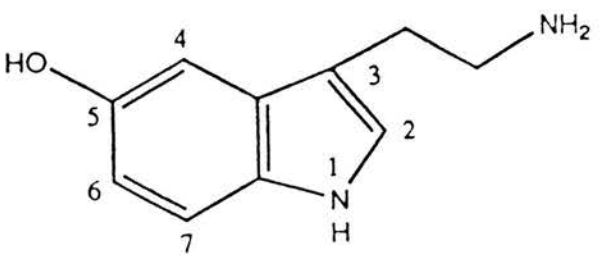


Table 3.1 Summary of the Indoleamines functional groupings, EC_{50} values and maximal stimulation values (n=4).

containing a methoxy group at R_2 and an acetyl grouping at R_1, demonstrated a small rise in aldsoertone secretion which was not significant. 5-hydroxytryptophol, with an hydroxy grouping at R_2 and at R_1, demonstrated no aldosterone stimulating activity. The compound 5-fluorotryptamine, with a fluorine grouping at R_2 and an amine grouping at R_1, caused a slight non-significant rise in aldosterone secretion. In contrast the compound 6-fluorotryptamine, with no R_2 grouping, and now a fluorine grouping at C_6, had no effect on aldosterone secretion. The compounds 5,6-dihydroxyltryptamine and 5.7-dihydroxyltryptamine, with no R_1grouping and now two hydroxy groupings at R_2, demonstrated no aldosterone stimulating activity.

cAMP secretion into the medium was also measured for the compounds that caused an equivalent aldosterone secretion to that of 5-HT, although due to the amount of cells required per experiment and problems with the cAMP assay itself, the experiments were only carried out once. cAMP as the second messenger for 5-HT stimulated aldosterone secretion has been proven by many groups, although cAMP secretion induced by the indoleamines studied here has not been fully evaluated to date. Our results largely agree with the previous data already published, and also with a previous study carried out in our laboratory by M. Hughes. The cAMP response to 5-HT was maximal at 10^{-7}M, following the same pattern as aldosterone secretion stimulated by 5-HT (Figure 3.8.). A cAMP response to 5-MeOT, N-methyltryptamine and 5-methyltryptamine was also observed (data not shown).

3.4. Discussion.

The molecule 5-HT, or 5-hydroxy-3-(2-aminoethyl) indole, consists of a diffuse 10 electron cloud above and below the heteroaromatic plane which is pulled towards the aminoethyl group at position 3 making this the most basic region of the molecule. At pH 7.5 the molecule is completely protonated (cationic form) whilst at pH 10.5 approximately 30% each of the neutral form and the zwitterionic form

exists. At alkaline pH, 5-HT in solution is rapidly destroyed when heated and because of this instability, 5-HT is sold commercially as the creatinine sulphate salt. At low pH, 5-HT is stable even at elevated temperatures, and can exist also as one of three stable salts, the picrate, hydrochloride or salicylate salt. Three different dissociation constants have been determined related to the creatinine moiety (pKa 4.9), the terminal amino group (pKa 10) and the hydroxy group within the benzyl ring (pKa 11.1).

The results obtained in this study are broadly consistent with the published investigations of indoleamine activity in stimulating aldosterone secretion. 5-HT and related compounds are subject to a variety of metabolic transformations, and so there exists a large and diverse group of naturally occurring indoleamines. In this study the following naturally occurring indoleamines were compared with 5-HT, L-tryptophan, tryptamine, melatonin, 5-HIAA, 5-HTP, N-methyltryptamine, 5-Methyltryptamine, 5-hydroxytryptophol and 5-methoxytryptamine.

Two of the naturally occurring compounds displayed similar strong agonist activity as compared to 5-HT, that is 5-MeOT and N-methyltryptamine. The response to 5-MeOT was equivalent to that of 5-HT. This response within perifused adrenal slices has also been reported by Contesse *et al.*, (1999). N-methyltryptamine also produced a strong stimulation of aldosterone secretion, although the response was different to that produced with 5-HT (Figure 3.3.), with a sharp rise from basal to maximal aldosterone secretion. This form of response would appear anomolous if the mechanism of this activation is a simple interaction of a single drug molecule with a receptor. It must be asked therefore whether this compound produces the stimulation seen in the same way as 5-HT. It is a unique compound amongst the various ones tested as it contains a small substituent, which improves the basicity of the terminal amine group, and this may result in an alteration in receptor binding providing the steep dose response relationship seen.

Tryptamine caused a high maximal stimulation of aldosterone, although the relative potency of this compound was low as seen with the high EC_{50} value recorded, consistent with a low-affinity, high-efficacy interaction with the receptor. Interestingly, Vane (1959) reported that the activity of tryptamine in rat stomach preparations was enhanced over 12 fold by the addition of an amine oxidase inhibitor, providing a greater activity in this preparation as compared to 5-HT. It was suggested that tryptamine had access to the intracellular oxidases, but that this access was denied to 5-HT, due to polar substituent groups preventing diffusion across the membrane. Should this oxidation be a factor in the ZG response to indoleamines, it may be that 10^{-7}M tryptamine is needed to saturate an oxidase, and that only above this concentration are agonist effects evident. It remains to be shown whether amine oxidase inhibition has an effect on tryptamine potency in this system. Interestingly, Contesse *et al.*, (1999), studied the ability of tryptamine (1nM to 10μM) to stimulate aldosterone secretion from perifused rat adrenocortical slices, and found that it was >1500 times less potent than 5-HT at eliciting a response.

5-methyltryptamine, 5-HTP and melatonin produced stimulation of aldosterone secretion, although the responses to these agonists were quite different. Melatonin caused very slight, non-significant stimulation of aldosterone secretion. 5-methyltryptamine on the other hand produced a significant stimulation of aldosterone, and the maximal stimulation seen was comparable to that seen with 5-HT (Figure 3.3). This occurred at a higher agonist concentration thus leading to a high EC_{50} value and a low relative potency. Although in isolation this effect may be attributed to a low receptor affinity, the potential affects of monoamine oxidase activity must also be considered. 5-HTP displayed a slight non-significant stimulation of aldosterone secretion at high concentrations of agonist. This effect may in fact be due to the slow *in vitro* formation of 5-HT from 5-HTP, and not in

fact due to 5-HTP itself. This effect will be discussed in greater detail in Chapter Five.

5-HIAA, 5-hydroxytryptophol and L-tryptophan demonstrated no stimulation of aldosterone at all. Thus along the principal path of 5-HT metabolism, 5-HT itself is the only compound of significant activity at the zona glomerulosa cell receptor.

These results for the naturally occurring indoleamines suggest that in addition to 5-HT, other indoleamines, synthesised constituitively by mammals, are equally capable of activating aldosterone synthesis. 5-MeOT, a compound that produced equivalent stimulation of aldosterone secretion as that due to 5-HT is found in plasma and may play an important role in aldosterone secretion. More investigations in to the physiological availability of these compounds are needed before any of the above agonists can be identified as potential physiological stimulants of aldosterone secretion.

To supplement the data from the naturally occurring compounds five synthetic compounds were looked at namely, 6-MeOT, 6-FT, 5-FT, 5,7DHT and 5,6DHT. The response to 6-MeOT indicated partial agonist activity (Figure 3.2.). The other synthetic compounds produced no significant stimulation.

From the observations in this study the substituent at the 5-position has little effect on the potency of the resulting molecule. This supports the observation of a "bulk tolerance" at the C_5 in binding studies at the pharmacologically similar brain 5-HT_2 receptor (Lyon, 1988; Bishop, 1990). The effect of exchanging a C_5 group for the same group at C_6 was studied by comparing 5-MeOT and 6-MeOT, and 5-FT with 6-FT. It was found in both cases that the activity of the 6-substituted compound was lower. This observation is supported by results from several other receptor types (Vane, 1959; Engel, 1983; Nichols, 1988; Lyon, 1988). The fact that this

trend appears to be irrespective of the electrostatic changes suggests that there is a high steric stringency imposed on C_6 substitutions. The complete inactivity of both the dihydroxytryptamines could equally be due to steric or electrosteric effects, so these results yield no significant information about the pharmacophore. Variations on the alkylamine side chain were also investigated. 5-HT has a pKa of 10, and so the terminal amine group is predominantly protonated at physiological pH (Vane, 1959). The existence of a positive charge on the terminal amine indicates that this group is particularly likely to be involved in binding receptors (Greenberg, 1960). It is therefore not surprising that the congeners in which this amine is missing, 5-HIAA and 5-hydroxytryptophol demonstrate no agonist activity. The compound 5-HTP, a chain substituted compound with a reduced basicity of this amine group demonstrated weak stimulating activity.

From table 3.1. it can be clearly seen that the indoleamines with a free group at position C_9 or with a methyl group attached to the nitrogen (N) atom are the most potent stimuli of aldosterone secretion. Thus, increasing the basic character at this position and thus the electron cloud negativity appears to be of primary importance in steroidogenic activity. Substitution, as seen in 5-MeOT, where the molecule still retains the amino portion, but now contains an additional oxygen grouping producing a more basic molecule, the potency of indoleamine stimulated aldosterone secretion is not affected, and in fact is equivalent to that seen with 5-HT. Substitution with a non-basic group as in 5-methyltryptamine, or indeed complete removal of the group at position C_5, for example tryptamine, slightly reduces the potency of stimulation, but not significantly. Thus the indole moeity appears to be of primary importance in stimulation of aldosterone synthesis. Modification of this region as in 5-hydroxytryptophol, gives rise to a compound that produces no significant rise in aldosterone secretion, and with melatonin, containing a 5-methoxy grouping and an N-acetyl grouping, conferring reduced basicity to the molecule, produced a slight non-significant rise in aldosterone.

Structure activity relationships for 5-HT and related compounds have been used in most studies investigating receptor types in certain tissues. Initially many groups believed that the receptor type present within the ZG was a "5-HT_{1c}/5-HT_2 like" receptor, the problem being that the second messenger system is cAMP within the ZG and not the PI system which is classically linked to these receptors (Mantero *et al.*, 1982; Matsuoko *et al.*, 1985; Maestri *et al.*, 1988). Now, in the frog and human gland, the receptor type has been shown to be a 5-HT_4 receptor (Lefebvre *et al.*, 1992; 1996), although in our studies with the rat adrenal gland this receptor type does not fit, and preliminary studies so far would give rise to the possibility that in the rat ZG the receptor type is either a 5-HT_6 or 5-HT_7 receptor, both of these are positively linked to cAMP, and these studies will be discussed in Chapter Five. From these structure activity studies, the requirement of an intact ethylamine structure would tend to suggest that if a 5-HT type receptor exists within the ZG, it requires this group for steroidogenesis as opposed to binding *per se*. Previously, it had been reported in studies by Niles *et al.*, (1983), that the hydroxyl group possessed by 5-HT and N-acetyl5-HT were essential for high affinity binding in the rat brain and since N-acetyl5-HT interacted with the 5-HT receptor, it was suggested that there was a close interaction on the same 5-HT receptor, however Cohen *et al.*, (1985) demonstrated that tryptamine could interact with 5-HT receptors in the rat fundic region, so the conformational requirement for stimulating through 5-HT receptors does not appear to be to rigid. Bennett & Snyder (1976), demonstrated in a series of binding studies in brain tissue, that the key requirement for 5-HT_1 high affinity binding was the 5-hydroxy substituted indole moiety. This idea is supported by studies carried out by Allgren *et al.*, (1985), in solubilised bovine cortical brain membranes. This study also observed that membranes specifically required potent interactions between the 5-hydroxy group and a free amino terminal group for high affinity binding. This effect was lost on solubilising the membranes. From this Allgren postulated that the 5-HT_1 site may exist as a

complex subunit containing separate amine and indole attachment sites. From the data in this study it could be suggested that the receptor present within the rat ZG is of the type 1 class, as previously thought. Perhaps throughout the 5-HT receptor classes different structural features and characteristics are necessary for binding to the individual receptor types. More studies with specific antagonists and agonists are needed before definitive characterisation of this receptor can be carried out.

Definitive proof of the existence of receptors within the ZG requires not only pharmacological studies, molecular biology studies and ligand binding studies, but also the existence of a second messenger system which acts as a coupling mechanism between initial hormone/receptor interaction and end steroid response, thus enabling the transformation of an initially small signal to a large end response by a complex cascade mechanism. The second messenger systems to all the major regulators of aldosterone, including 5-HT, have already been studied in great detail and are discussed fully in Chapter 1. The results shown clearly illustrate that 5-HT, as previously published, stimulates cAMP production, indicating that the receptor type present in the ZG of the rat adrenal gland is positively coupled to cAMP. Of the other compounds that stimulated aldosterone secretion, cAMP production was also stimulated. The increase seen may in fact be due to contaminating ZF cells, although this is highly unlikely as the steroidogenic capacity of 5-HT is confined to the ZG. However, in the case of the other stimulatory compounds, this fact has not been proven and further studies into the action of these compounds on the ZF must be carried out before a complete picture of these compounds within the adrenal gland can be formed. Of interest is the fact that the concentration of the compounds, including 5-HT, required to stimulate cAMP was much higher than that needed to induce steroidogenesis, indicating a clear dissociation between second messenger and end response. A similar effect was observed for ACTH in adrenocortical cells obtained from decapsulated adrenal glands by other groups who demonstrated a discrepancy between adenylate cyclase activity and

steroidogenesis. They also reported that specific binding of ACTH to cells correlates well with cAMP production but not steroidogenesis (Beall & Sayers, 1972; Mackie *et al.*, 1972). These results strongly support the receptor reserve model by Hornsby & Gill (1978), which suggested that only a small fraction of cAMP formed in response to hormonal stimulation is required for steroidogenesis.

Further studies are required to fully evaluate the importance of compounds such as 5-methoxytryptamine and tryptamine in aldosterone secretion. A better system for this evaluation would be the perfused adrenal capsule, allowing investigation of the whole tissue without the harsh collagenase digestion process involved in the formation of isolated ZG cells. However, capsular preparations provide little or no response at low doses of compound due to the inaccessibility of cell surface receptors. Additivity studies, in which the compounds that did not stimulate aldosterone secretion are incubated with 5-HT, would be useful in establishing whether or not those compounds were in fact having an antagonistic effect at the receptor. The effects of salt diets on aldosterone secretion could also be looked at, also the effects of amine oxidase must also be looked at in more detail.

These structure-activity studies give preliminary indications of the functional groupings important for indoleamine interaction with the zona glomerulosa cell receptor. It would appear that the 5-hydroxyl group is not a requirement for full agonist activity, although substitution, but that ring substitutions at other positions may compromise that activity. The basicity of the terminal amine group appears also to be important in receptor binding. Compounds lacking both a basic terminal amine grouping and an hydroxy, methyl or hydroxymethyl grouping at C_5, lack aldosterone stimulating activity.

Chapter Four

Pharmacological Characterisation of 5-HT Receptors in the Rat Adrenal Zona Glomerulosa.

4.1. Introduction.

Functionally, 5-HT is a signalling molecule, acting throughout the body in various ways. Numerous receptors exist for 5-HT throughout the body, often found in areas where there is no apparent nearby neuronal or autocrine/endocrine source of 5-HT supply. Over the past few years a number of "new" 5-HT receptors have been discovered, so that at the moment seven different classes of 5-HT receptor exist, which have been discussed in detail in Chapter One.

Although, as yet there is no clearly defined physiological role for 5-HT in aldosterone secretion, *in vitro* it remains one of the most potent stimulators of aldosterone secretion from the ZG, although the functional and physiological relevance of this still remains to be elucidated. Since the first discovery of the stimulatory capacity of 5-HT on the ZG of a variety of species, many studies have been carried out to determine the 5-HT receptor site within the adrenal cortex. Historically, from studies utilising ketanserin and cyproheptadine, in the rat, it was proposed that 5-HT-induced aldosterone production was mediated through activation of a 5-HT_2 receptor. Classically these receptors are linked to the stimulation of phospholipase C. In a study by Rocco *et al.*, (1990), no influence of 5-HT on inositol phosphate formation was observed from isolated rat ZG cells stimulated with 5-HT, indicating that the steroidogenic effect of 5-HT within the rat ZG is not mediated through 5-HT_2 receptors. Moreover, the second messenger for 5-HT stimulated aldosterone secretion within the ZG is cAMP (Matsuoka *et al.*, 1985; Maestri *et al.*, 1988). With the discovery of more 5-HT receptor families, and more agonists, antagonists and radioligands, it has been possible to identify the receptor site within the adrenal cortex of various species. In one study, it was reported that the 5-HT_4 receptor, positively coupled to cAMP, is present both in the human and frog adrenal gland (Lefebvre *et al.*, 1993). Also, in one study in the hamster using a probe for the 5-HT_6 receptor, a faint signal was found to be present

within the adrenal cortex (Ruat *et al.*, 1993). In the rat, certain antagonists for the 5-HT_6 and 5-HT_7 receptors have previously been shown to be the most effective antagonists for inhibition of 5-HT stimulated aldosterone secretion from isolated rat zona glomerulosa cells (Lisa Bishop, Honours Project, 1989). From these various studies the corticotropic effect of 5-HT was conserved, however the 5-HT receptor site differs from species to species. In fact Contesse *et al.*, (1999), finally characterised the rat receptor site within the ZG as the 5-HT_7 receptor, positively coupled to cAMP.

A link between sodium status and aldosterone secretion was first reported over 40 years ago. It is now apparent that sodium status is one of the most potent regulators of aldosterone secretion. In a sodium deplete state the ZG undergoes morphological changes, with an increase in the width of the ZG observed, also the responsiveness to certain factors is increased. The opposite effect happens in a sodium loaded tissue. The exact mechanism of this has remained largely unclear, due to the large number of physiological effects initiated by altered sodium status. For example, the RAS is affected. Lowering sodium increases renin secretion from the juxtaglomerular cells of the kidney, leading to an increase in AII receptors and subsequent increase in ZG responsiveness to AII. Also a decreased extracellular fluid volume, circulating plasma volume and arterial blood pressure, all activating the RAS, will be follow on effects of a sodium deplete state. Another factor would be retention of potassium ions which can stimulate aldosterone secretion directly. The RAS therefore plays an important role in the action of sodium levels on aldosterone secretion, although this is not the only factor involved. For example, it has been shown in nephrectomised animals, that during sodium restriction, there is an increase in aldosterone production. Some groups have suggested the involvement of a pituitary derived factor, not ACTH, which is secreted during sodium depletion and stimulates aldosterone production (McCaa *et al.*, 1974). Davis *et al.*, (1968), suggested a direct adrenal action of low sodium acting at both the

early and late stages of the biosynthetic pathway. Another factor is the adrenal RAS system, which may account for the effects seen, especially in nephrectomised animals (Doi *et al.*, 1984). If 5-HT acts physiologically to alter aldosterone secretion from the ZG, sodium status should also affect the actions of the indoleamine.

At the time of this work no definitive study had been carried out to characterise the 5-HT receptor type within the rat ZG. The aim of this study, therefore, was to define the 5-HT receptor type present within the zona glomerulosa of the rat adrenal gland, using a wide range of 5-HT agonists and antagonists, and studying their effect on aldosterone secretion from freshly prepared isolated rat zona glomerulosa cells. The effects of a change in sodium status in the rat was also investigated with the 5-HT agonists showing comparable stimulation of aldosterone as to that seen with 5-HT. This would add further evidence for a physiological role of 5-HT in aldosterone secretion, if salt intake modulated its effect.

4.2. Statistical Analysis.

Statistical significance was calculated in the individual experiments by Student's t-test for unpaired samples. A p value of <0.05 was considered significant. NS indicates non significance. *, ** and *** indicates that $p<0.05$, $p<0.01$ and $p<0.001$ respectively.

4.3. Results.

The graphs illustrated are the mean ± SEM of four individual experiments, within each experiment each incubation was repeated in triplicate. Figure 4.1 shows a typical aldosterone dose reponse to 5-HT (10^{-10}M-10^{-5}M) in the rat zona glomerulosa cell preparation. 10^{-8}M 5-HT caused a significant rise in aldosterone secretion, maximal stimulation occurred at 10^{-6}M, causing approximately a four to six fold increase in aldosterone output.

Figure 4.2 shows the aldosterone stimulation produced by the 5-HT_4 agonists BIMU 1 and BIMU 8. No significant stimulation of aldosterone from freshly prepared isolated rat zona glomerulosa cells was observed, at any concentration of both BIMU 8 and BIMU 1, as compared to 5-HT (n=4).

Figure 4.3 depicts dose response curves to the second class of 5-HT_4 agonists, the benzamide derivatives zacopride and cisapride. In our study however no significant stimulation of aldosterone from the rat zona glomerulosa was observed with these compounds at any concentration, as compared to 5-HT (n=4).

Figure 4.4. depicts the dose response to DAU 6285, a benzimidazolone derivative that acts as an antagonist at the 5-HT_4 receptor, in the presence of 10^{-7}M 5-HT (n=4). No significant inhibition of the stimulation of aldosterone induced by 5-HT within the rat zona glomerulosa was observed at any concentration.

From the results depicted in Figures 4.2, 4.3 and 4.4 the presence of a 5-HT_4 receptor within the rat zona glomerulosa seem unlikely.

Figure 4.5 represents the dose response to CV 205 502, a 5-HT_3 receptor antagonist, in the presence of 10^{-7}M 5-HT (n=4). No significant inhibition of 5-HT induced aldosterone secretion was observed at any concentration. This result would suggest that there are no 5-HT_3 receptors present within the rat zona glomerulosa.

Figure 4.6 represents the dose response to ketanserin, a 5-HT_2 receptor antagonist, in the presence of 10^{-7}M 5-HT (n=4). Significant inhibition of 5-HT induced aldosterone secretion was observed at 10^{-6}M and 10^{-5}M ketanserin. This result has previously been

observed by other workers, suggesting the presence of a 5-HT_2 receptor within the rat zona glomerulosa (Williams *et al.*, 1984; Matsuoka *et al.*, 1985). However the second messenger system classically linked to this receptor type is the PI system and it is now well established that the second messenger system for 5-HT induced secretion within the zona glomerulosa is cAMP (Fujita, 1979; Kojima *et al.*, 1982).

Figure 4.7 represents the dose response to mesulergine, a 5-HT_2 and 5-HT_7 receptor antagonist, with weak antagonistic properties at the 5-HT_6 receptor, in the presence of 10^{-7}M 5-HT (n=4). Significant inhibition of the aldosterone stimulation induced by 5-HT was observed at 10^{-7}M, 10^{-6}M and 10^{-5}M mesulergine. This effect again has been observed in previous studies (Maestri *et al.*, 1982; Lisa Bishop, Hons. Project, 1989). The second messenger system classically linked to the 5-HT_{1c} receptor is the PI system; however, in the case of both the 5-HT_6 and 5-HT_7 receptors the second messenger system is cAMP, a fact that supports the presence of either of these receptors within the rat zona glomerulosa.

Figure 4.8 represents the dose response to cyanopindolol, a 5-HT_{1a} and 5-HT_{1b} receptor antagonist, in the presence of 10^{-7}M 5-HT (n=4). Significant inhibition was observed at the higher concentration of this antagonist. However the 5-HT_1 receptor family is linked to the PI system, and the receptor site present within the rat ZG is positively linked to adenylyl cyclase, so it seems unlikely that a receptor belonging to the 5-HT_1 family is present within the rat ZG.

Figure 4.9 depicts ICS 205/930, an indoleamine derivative, a 5-HT_3 and 5-HT_4 receptor antagonist, in the presence of 10^{-7}M 5-HT. No significant inhibition was observed at any concentration of this antagonist, suggesting that these receptor types are not present within the rat zona glomerulosa (n=4). This compound within the frog and

human adrenal gland produced inhibition of 5-HT, zacopride, cisapride, BIMU 1 and BIMU 8 (Lefebvre *et al.*, 1992; Idres *et al.*, 1991). The results for the antagonists are summarised in Figure 4.10. The % inhibition for the antagonists are summarised in Table 4.2.

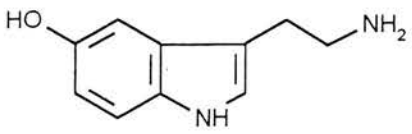
Figure 4.11, represents dose responses to the agonists 5-methoxytryptamine and 5-carboxamidotryptamine, as compared to 5-HT (n=3). These agonists are indole derivatives fairly selective for the 5-HT_4, 5-HT_6 and 5-HT_7 receptor sites, however 5-CT only produces comparable aldosterone stimulation to that of 5-HT at very high concentrations at the 5-HT_4 receptor site. As can be seen the agonists produce similar cells. These data strongly support the presence of either a 5-HT_6 or 5-HT_7 receptor site within the rat zona glomerulosa, although the presence of a "5-HT_{1c}/5-HT_2" like receptor with cAMP as its second messenger as opposed to inositol phosphate can not be ruled out.

The effects of sodium status, on the aldosterone stimulating abilities of these indole agonists, are shown, in Figure 4.12 (n=3). Sodium status is probably the main regulator of aldosterone secretion, and is discussed in Chapter One. Animals kept on a low sodium diet (0%), a high sodium diet (3%), or a normal sodium diet (1%), for one week, were sacrificed by cervical dislocation and their adrenals removed and isolated zona glomerulosa cells prepared. The aldosterone dose response curves to all three agonists show no significant difference within any particular salt diet; however a significant difference between salt diets clearly exists ($p<0.05$). Responses from low salt diets are highest, conversely, high salt diets have the lowest aldosterone output. Figures 4.13, 4.14 and 4.15 depict the dose response to mesulergine, in the presence of 10^{-7}M 5-HT, 5-methoxytryptamine and 5-carboxamidotryptamine respectively, from animals from all three salt diet groups (n=3). Mesulergine resulted in a dose dependent

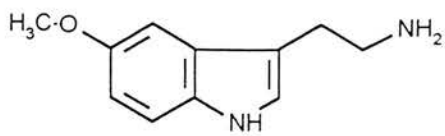
Graphical Illustration

- Chapter Four -

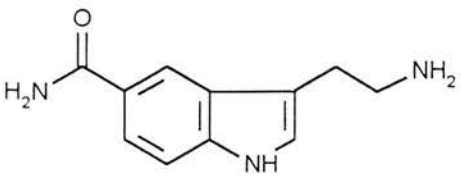
STRUCRURES OF 5-HT RECEPTOR AGONISTS



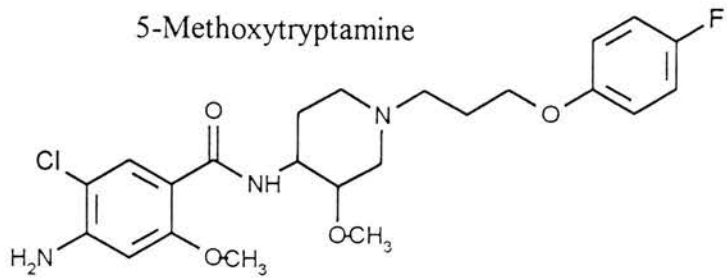
5-Hydroxytryptamine (5-HT)



5-Methoxytryptamine



5-Carboxamidotryptamine



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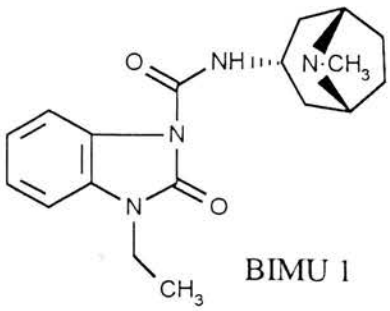
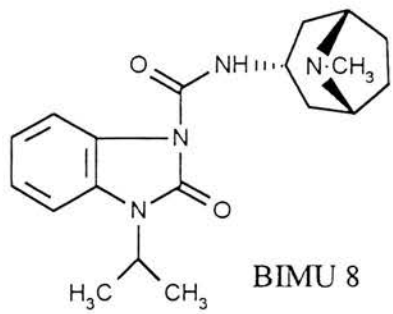


BIMU 1



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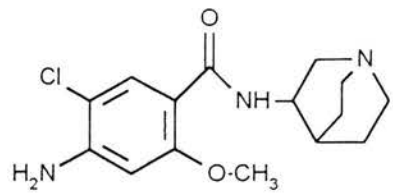
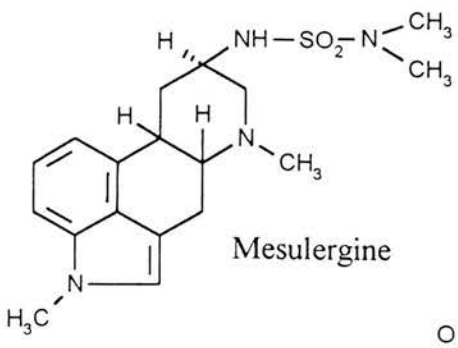


Zacopride

STRUCTURES OF 5-HT RECEPTOR ANTAGONISTS



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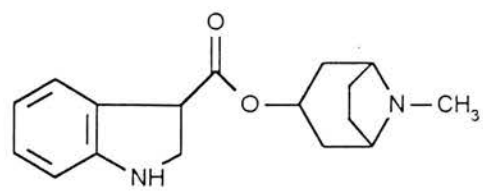
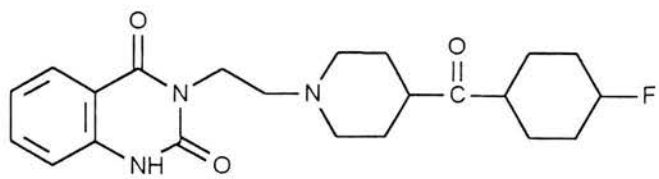


ICS 205,930



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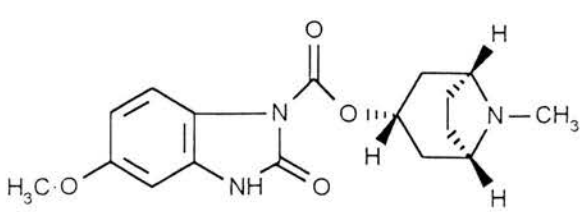

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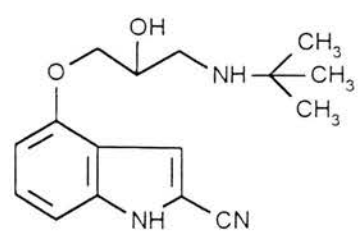

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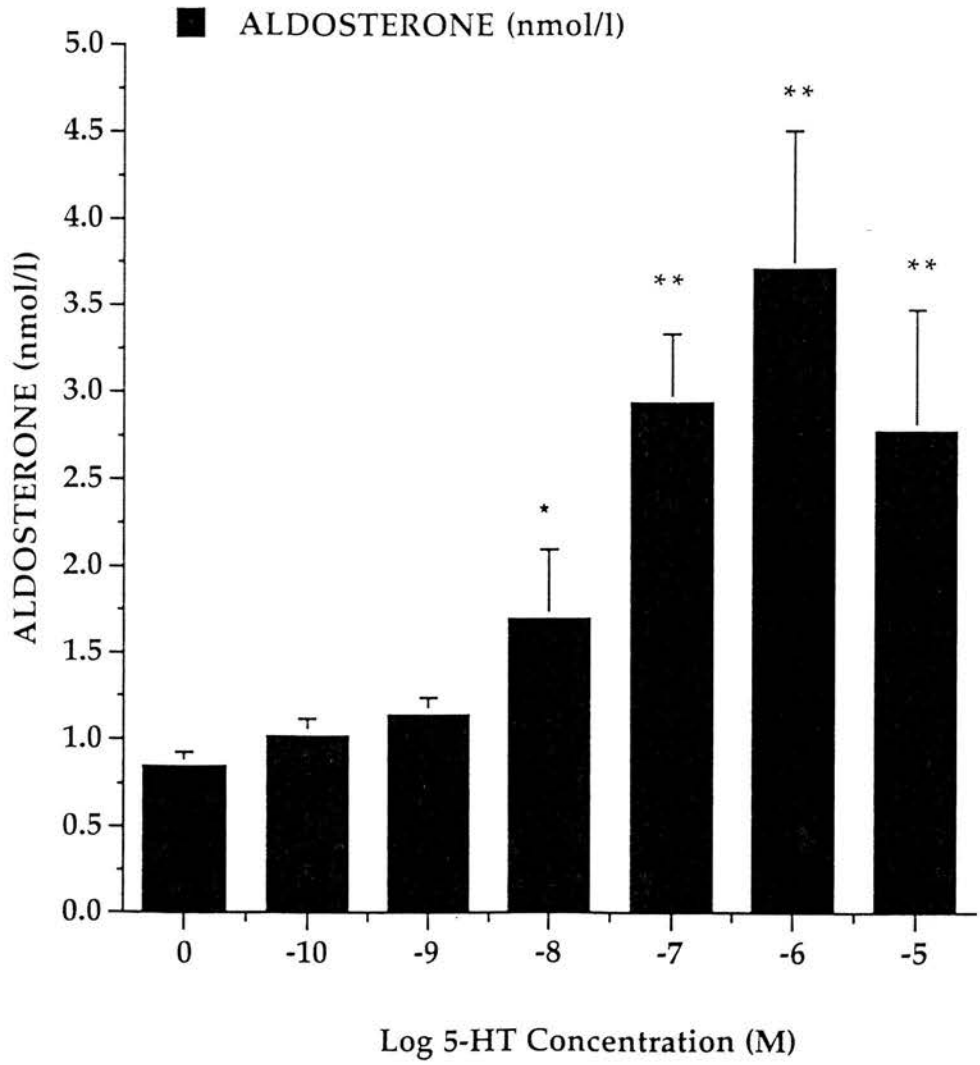


Figure 4.1. *Dose-dependent increase in aldosterone secretion from isolated ZG cells in response to increasing concentrations of 5-HT.* Isolated rat ZG cells were incubated with increasing concentrations of 5-HT for 1 hour at 37°C. Aldosterone secretion into the medium was measured by RIA. Data from 4 separate experiments, with triplicate incubations performed in each, were combined and expressed as mean±SEM. *P<0.05 and **p<0.01 compared to basal levels.

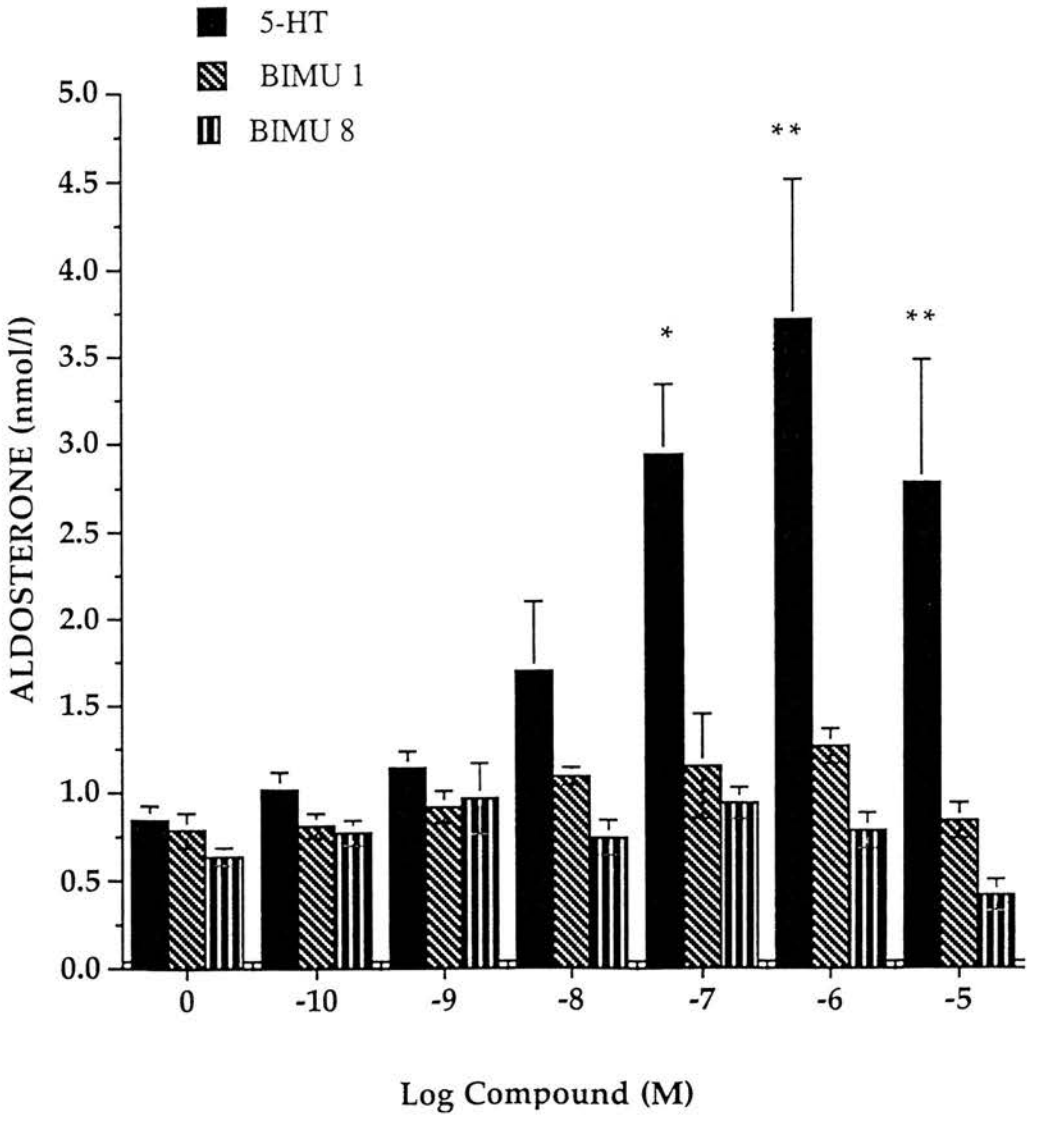


Figure 4.2. *Dose-dependent increase in aldosterone secretion from isolated ZG cells in response to increasing concentrations of 5-HT, BIMU1 and BIMU8.* Isolated rat ZG cells were incubated with increasing concentrations of 5-HT, BIMU1 or BIMU8 for 1 hour at 37°C. Aldosterone secretion into the medium was measured by RIA. Data from 4 separate experiments, with triplicate incubations performed in each, were combined and expressed as mean±SEM. *P<0.05 and **p<0.01 compared to basal levels.

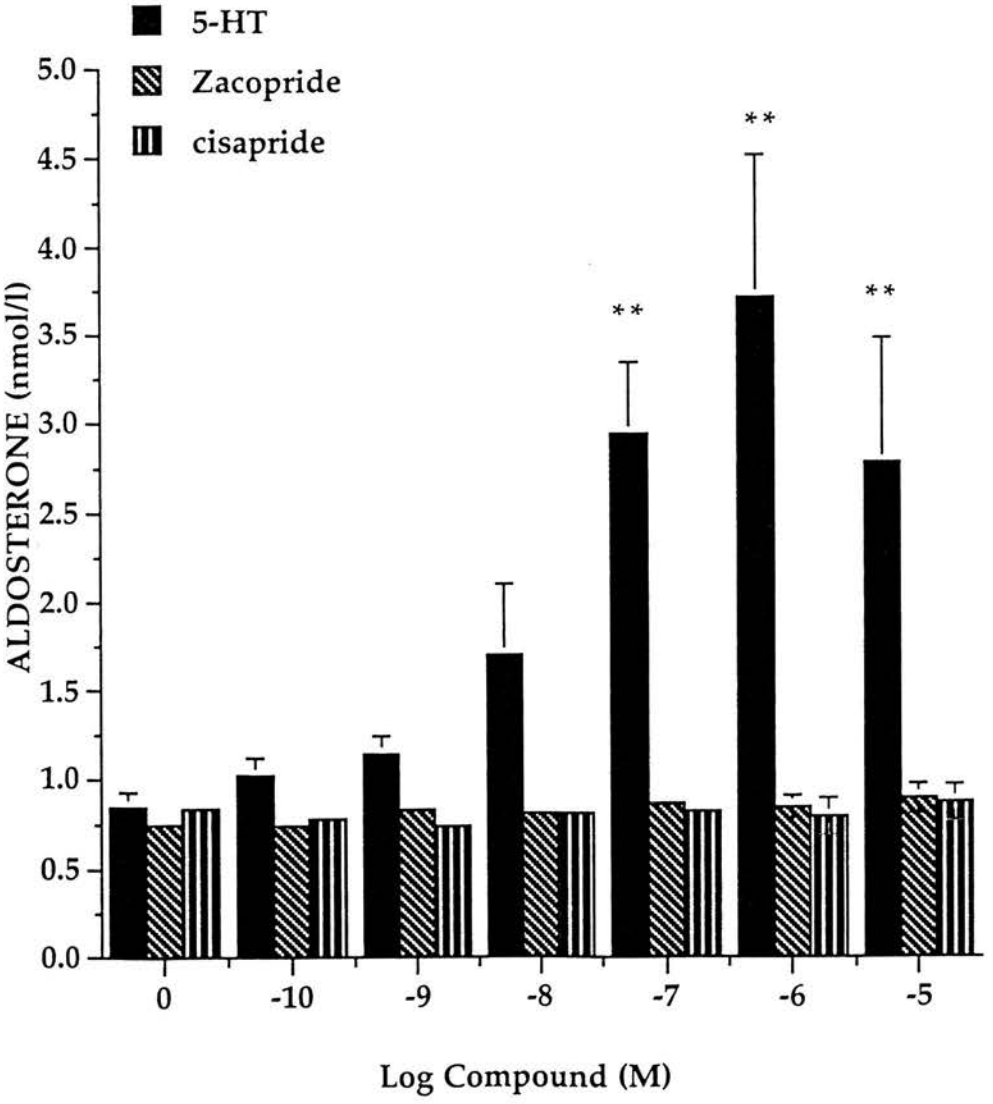


Figure 4.3. *Dose-dependent increase in aldosterone secretion from isolated ZG cells in response to increasing concentrations of 5-HT, zacopride and cisapride.* Isolated rat ZG cells were incubated with increasing concentrations of 5-HT, zacopride or cisapride for 1 hour at 37°C. Aldosterone secretion into the medium was measured by RIA. Data from 4 separate experiments, with triplicate incubations performed in each, were combined and expressed as mean±SEM. *P<0.05 and **p<0.01 compared to basal levels.

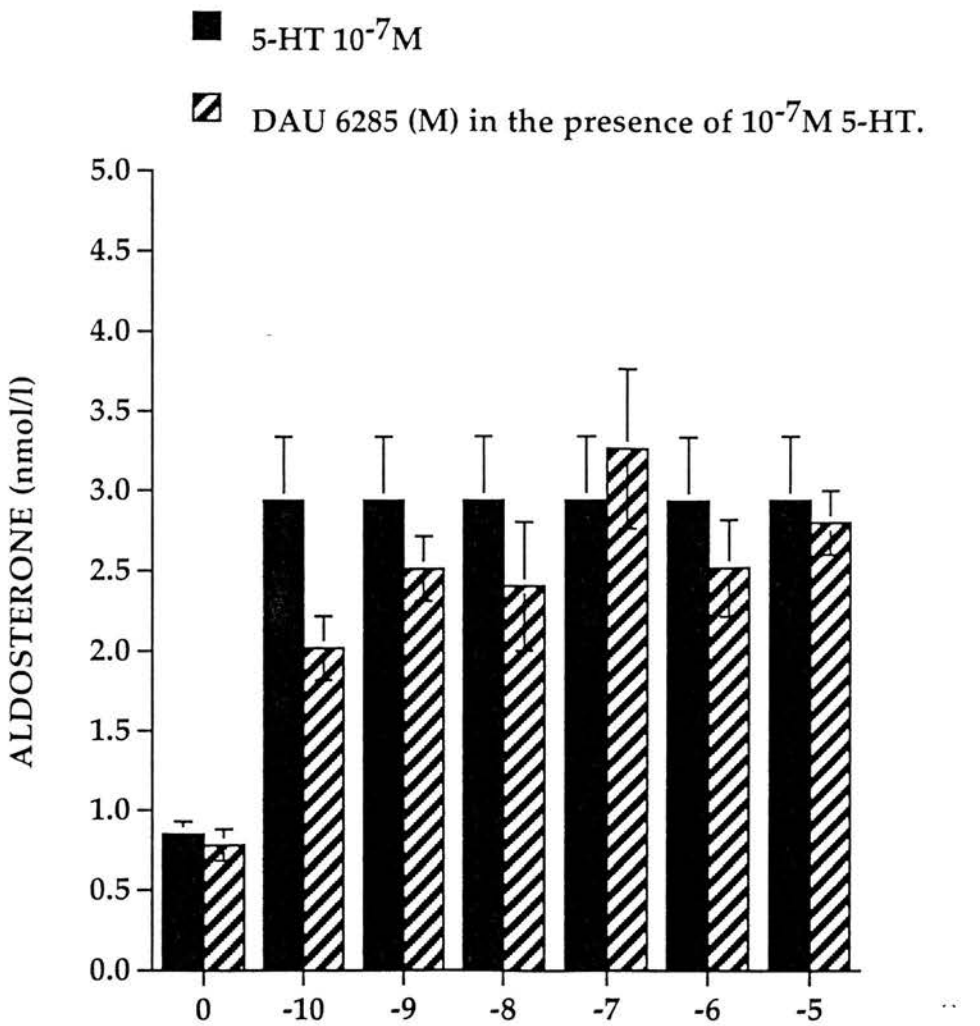


Figure 4.4. *Aldosterone secretion from isolated ZG cells in response to 5-HT 10^{-7}M in the presence of increasing concentrations of DAU 6285.* Isolated rat ZG cells were incubated with 5-HT at 10^{-7}M in the presence of increasing concentrations of DAU 6285 for 1 hour at 37°C. Dose 0 reflects basal aldosterone secretion into the medium, in the presence and absence of DAU 6285 (10^{-7}M). Aldosterone secretion into the medium was measured by RIA. Data from 4 separate experiments, with triplicate incubations performed in each, were combined and expressed as mean±SEM.

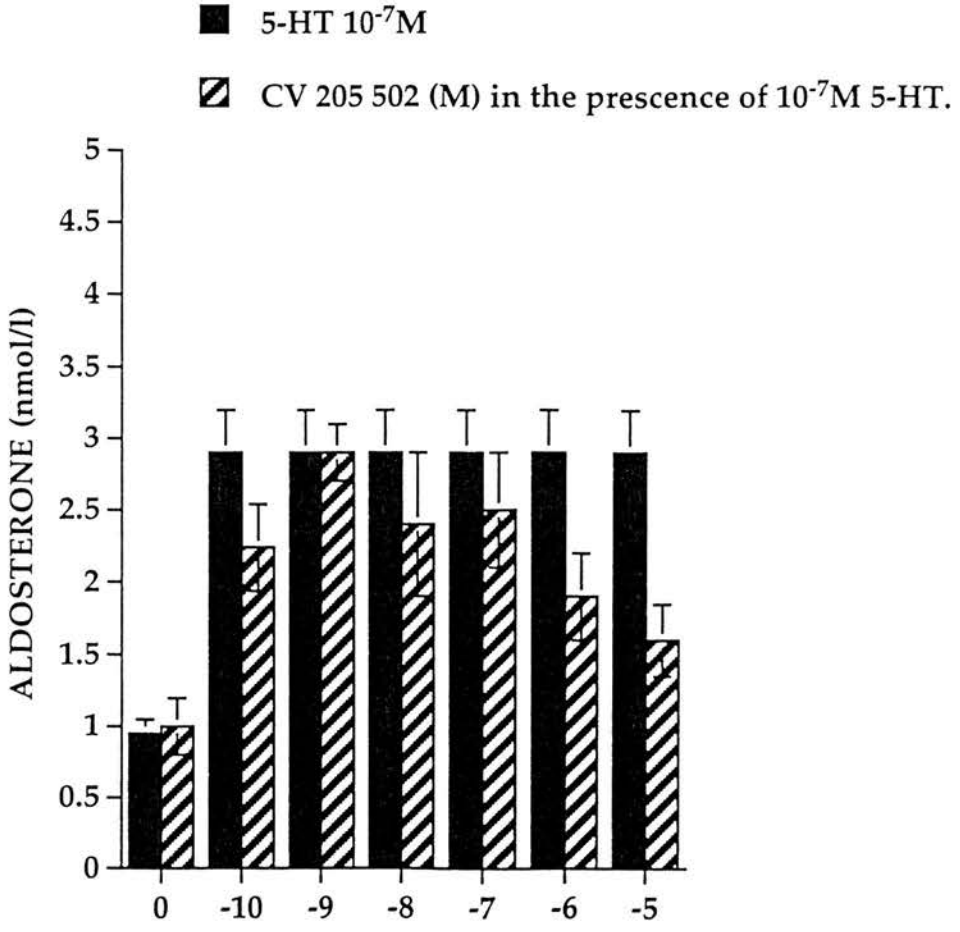


Figure 4.5. *Aldosterone secretion from isolated ZG cells in response to 5-HT 10^{-7}M in the presence of increasing concentrations of CV 205 502.* Isolated rat ZG cells were incubated with 5-HT at 10^{-7}M in the presence of increasing concentrations of CV 205 502 for 1 hour at 37°C. Dose 0 reflects basal aldosterone secretion into the medium, in the presence and absence of CV 205 502 (10^{-7}M). Aldosterone secretion into the medium was measured by RIA. Data from 4 separate experiments, with triplicate incubations performed in each, were combined and expressed as mean±SEM.

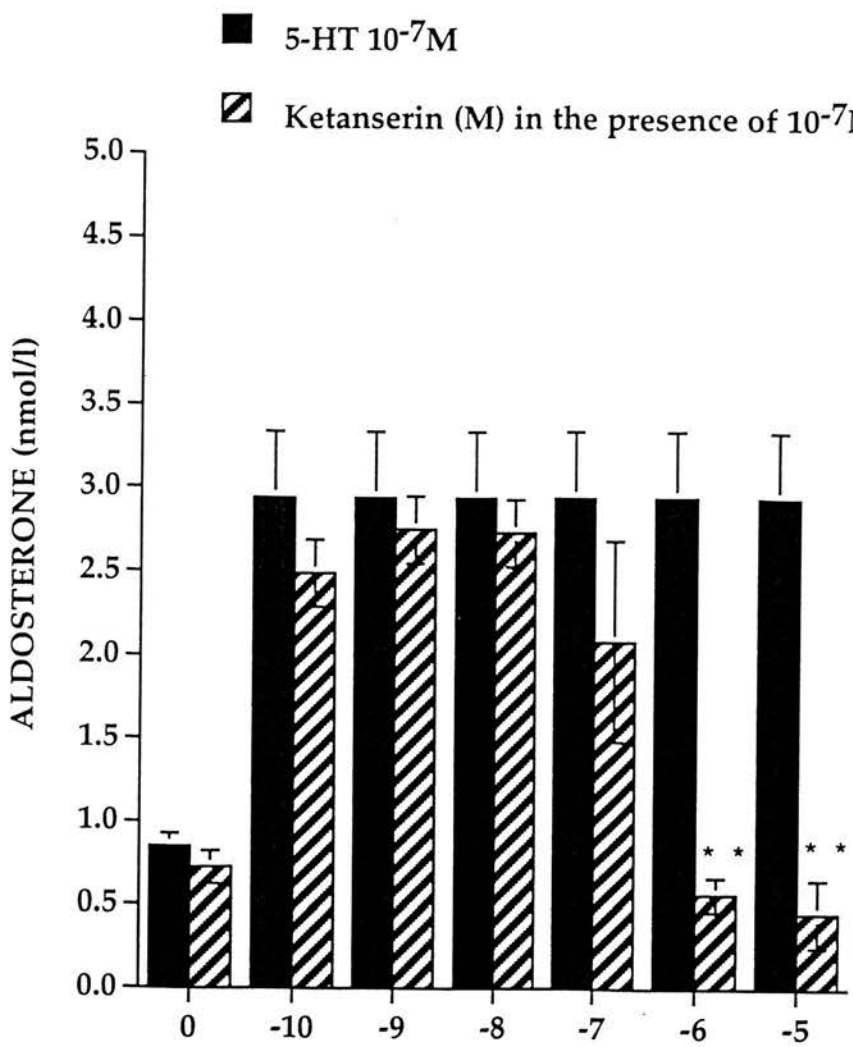


Figure 4.6. *Aldosterone secretion from isolated ZG cells in response to 5-HT 10^{-7}M in the presence of increasing concentrations of ketanserin.* Isolated rat ZG cells were incubated with 5-HT at 10^{-7}M in the presence of increasing concentrations of ketanserin for 1 hour at 37°C. Dose 0 reflects basal aldosterone secretion into the medium, in the presence and absence of ketanserin (10^{-7}M). Aldosterone secretion into the medium was measured by RIA. Data from 4 separate experiments, with triplicate incubations performed in each, were combined and expressed as mean±SEM. Statistically significant differences between 5-HT stimulated aldosterone secretion and 5-HT stimulated aldosterone secretion in the presence of an antagonist are indicated by **$p<0.01$.

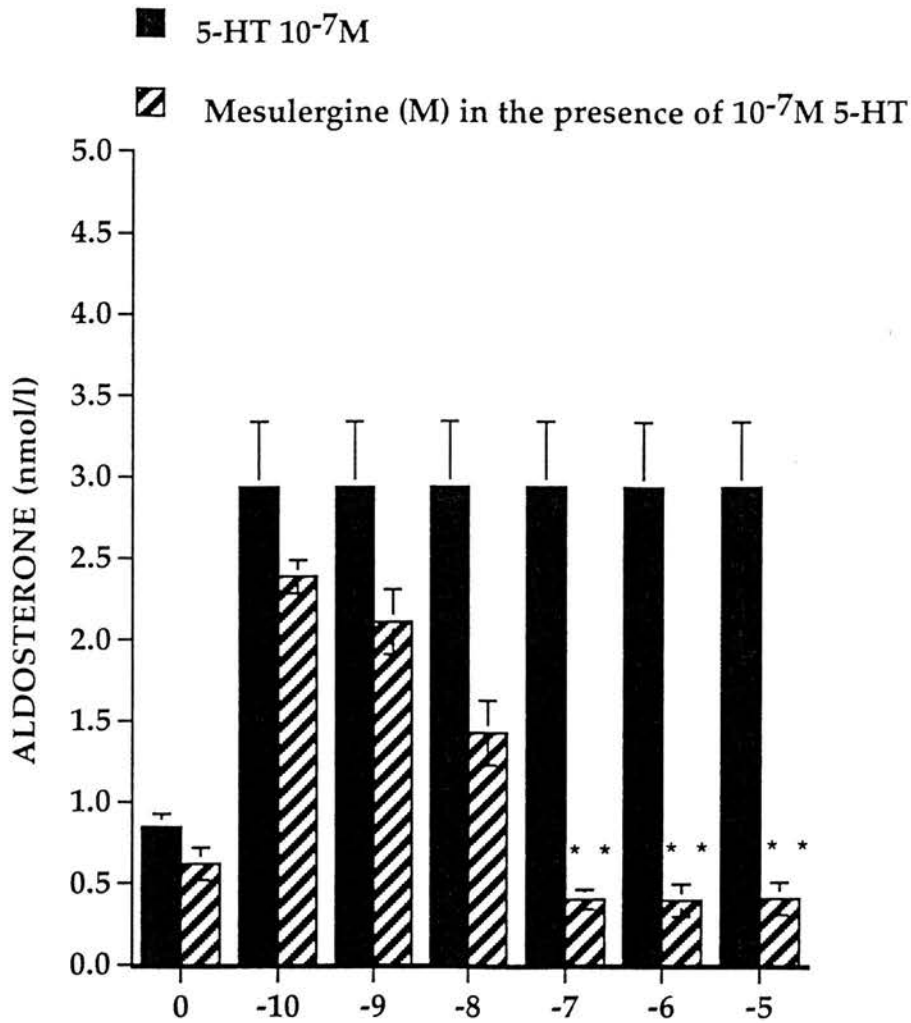


Figure 4.7. *Aldosterone secretion from isolated ZG cells in response to 5-HT 10^{-7}M in the presence of increasing concentrations of mesulergine.* Isolated rat ZG cells were incubated with 5-HT at 10^{-7}M in the presence of increasing concentrations of mesulergine for 1 hour at 37°C. Dose 0 reflects basal aldosterone secretion into the medium, in the presence and absence of mesulergine (10^{-7}M). Aldosterone secretion into the medium was measured by RIA. Data from 4 separate experiments, with triplicate incubations performed in each, were combined and expressed as mean±SEM. Statistically significant differences between 5-HT stimulated aldosterone secretion and 5-HT stimulated aldosterone secretion in the presence of an antagonist are indicated by **$p<0.01$.

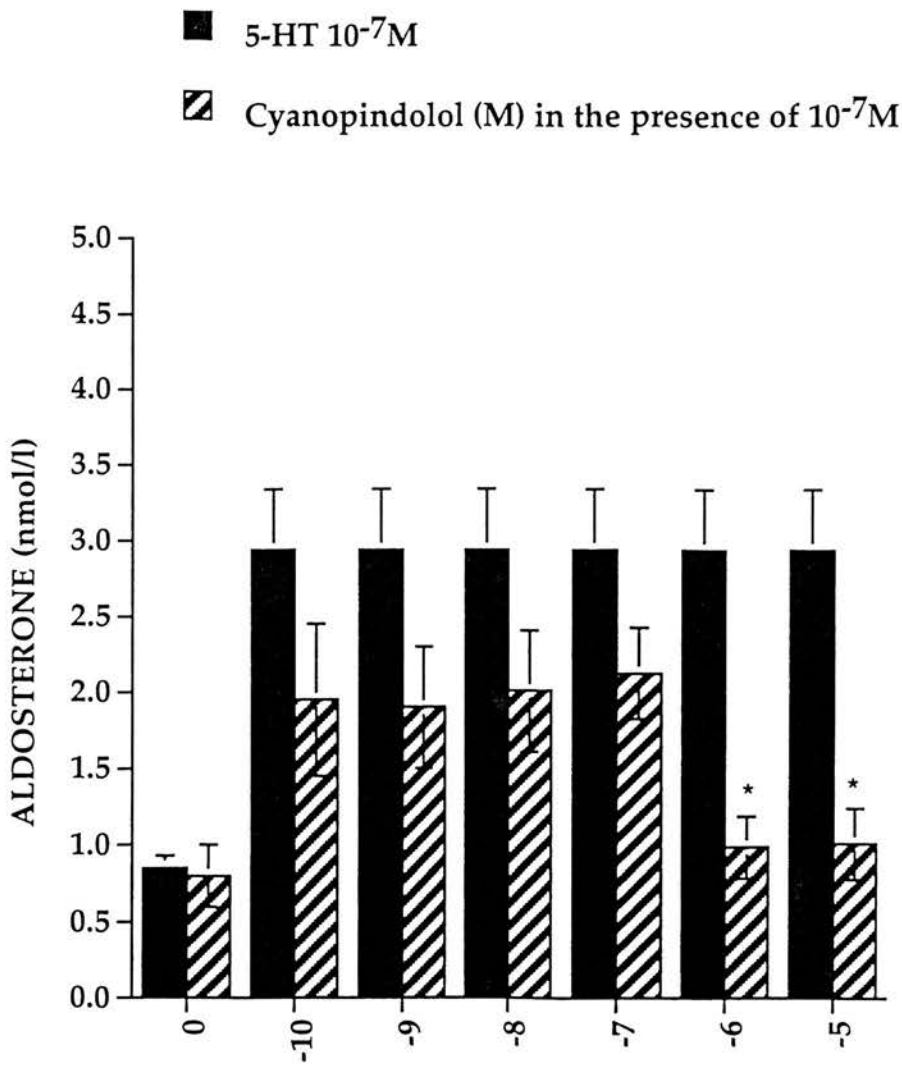


Figure 4.8. *Aldosterone secretion from isolated ZG cells in response to 5-HT 10^{-7}M in the presence of increasing concentrations of cyanopindolol.* Isolated rat ZG cells were incubated with 5-HT at 10^{-7}M in the presence of increasing concentrations of cyanopindolol for 1 hour at 37°C. Dose 0 reflects basal aldosterone secretion into the medium, in the presence and absence of cyanopindolol (10^{-7}M). Aldosterone secretion into the medium was measured by RIA. Data from 4 separate experiments, with triplicate incubations performed in each, were combined and expressed as mean±SEM. Statistically significant differences between 5-HT stimulated aldosterone secretion and 5-HT stimulated aldosterone secretion in the presence of an antagonist are indicated by *$p<0.05$.

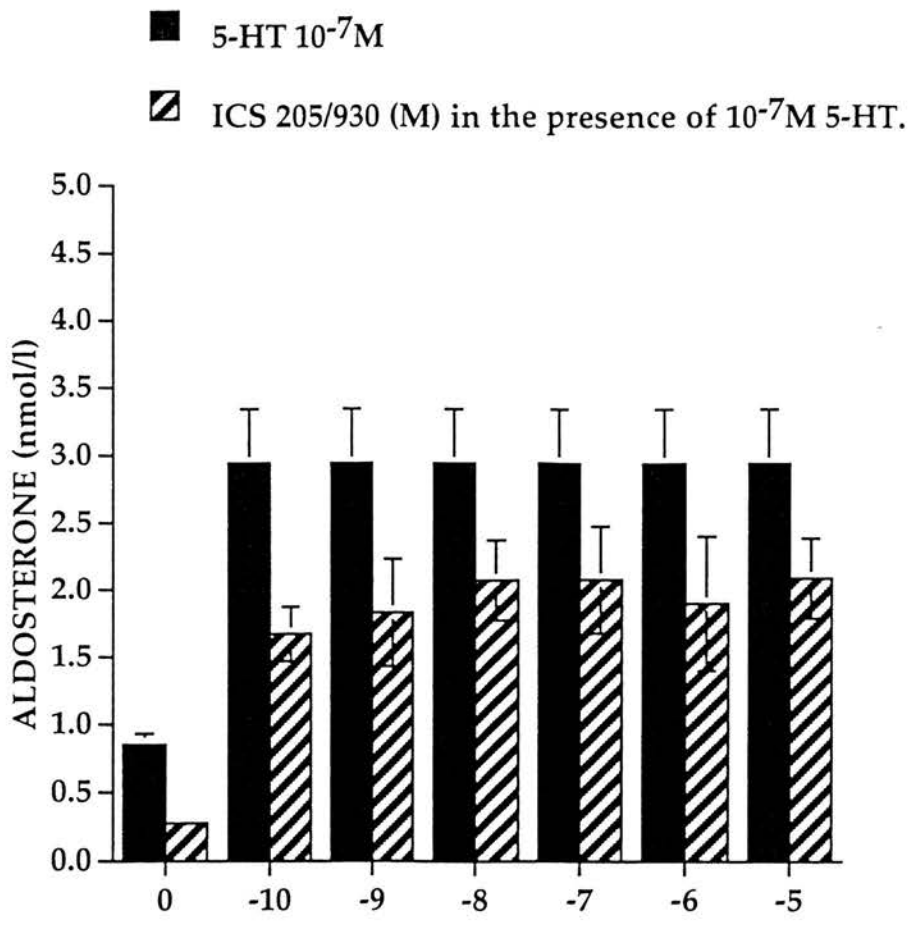


Figure 4.9. *Aldosterone secretion from isolated ZG cells in response to 5-HT 10^{-7}M in the presence of increasing concentrations of ICS 205/930.* Isolated rat ZG cells were incubated with 5-HT at 10^{-7}M in the presence of increasing concentrations of ICS 205/930 for 1 hour at 37°C. Dose 0 reflects basal aldosterone secretion into the medium, in the presence and absence of ICS 205/930 (10^{-7}M). Aldosterone secretion into the medium was measured by RIA. Data from 4 separate experiments, with triplicate incubations performed in each, were combined and expressed as mean±SEM.

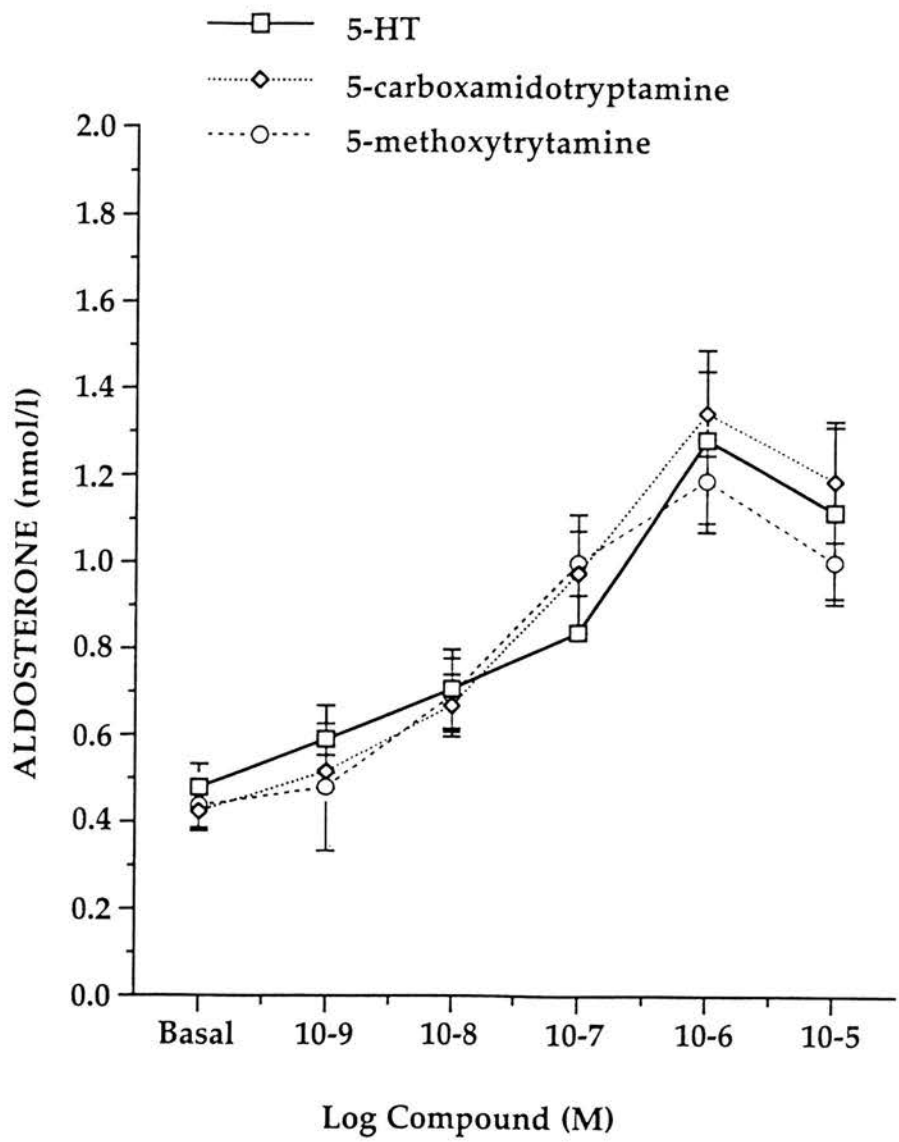


Figure 4.10. *Dose-dependent increase in aldosterone secretion from isolated ZG cells in response to increasing concentrations of 5-HT, 5-CT and 5-MeOT.* Isolated rat ZG cells were incubated with 5-HT, 5CT or 5-MeOT for 1 hour at 37°C. Aldosterone secretion into the medium was measured by RIA. Data from 4 separate experiments, with triplicate incubations performed in each, were combined and expressed as mean±SEM.

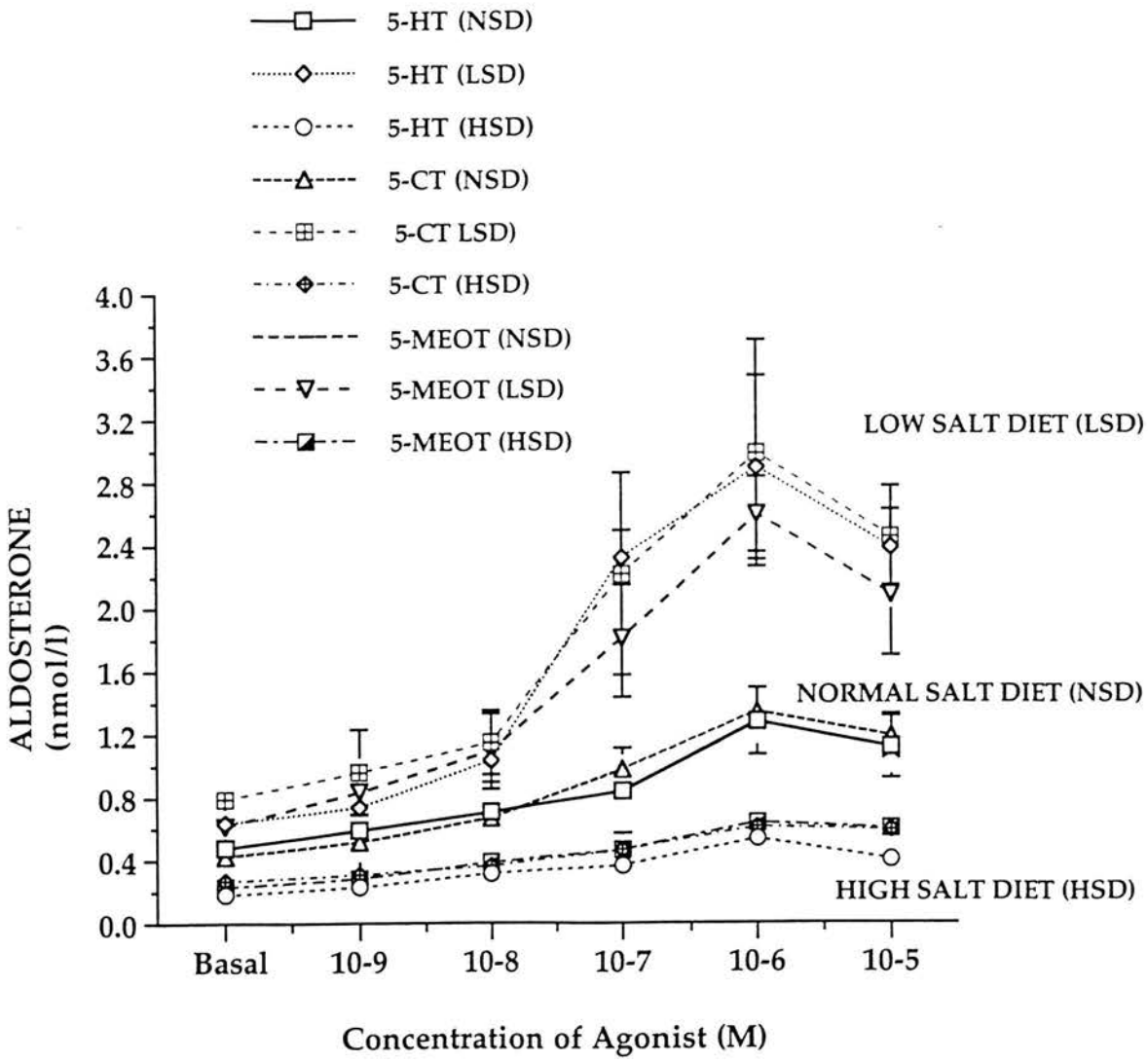


Figure 4.11. *Dose-dependent increase in aldosterone secretion from isolated ZG cells, prepared from rats fed on a normal, low and high salt diet, in response to increasing concentrations of 5-HT, 5-CT and 5-MeOT.* Female Wistar rats were fed on either a normal (1%), low (0%) or high (3%) salt diet for one week prior to removal of their adrenal glands. Isolated rat ZG cells were then prepared and incubated with 5-HT, 5CT or 5-MeOT for 1 hour at 37°C. Aldosterone secretion into the medium was measured by RIA. Data from 4 separate experiments, with triplicate incubations performed in each, were combined and expressed as mean±SEM.

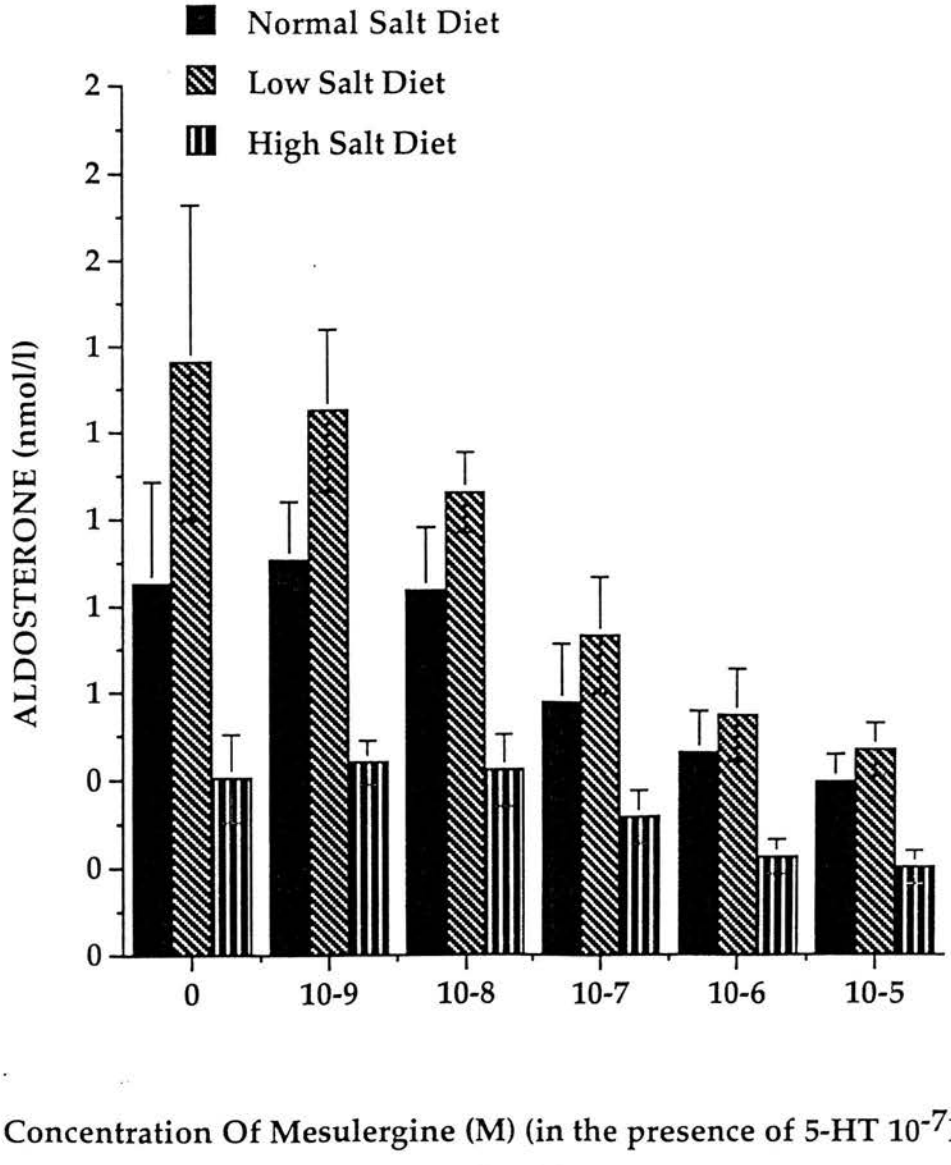


Figure 4.12. *Dose-dependent increase in aldosterone secretion from isolated ZG cells, prepared from rats fed on a normal, low and high salt diet, in response to increasing concentrations of mesulergine in the presence of 5-HT $10^{-7}M$.* Female Wistar rats were fed on either a normal (1%), low (0%) or high (3%) salt diet for one week prior to removal of their adrenal glands. Isolated rat ZG cells were then prepared and incubated with increasing concentrations of mesulergine in the presence of 5-HT 10^{-7}M for 1 hour at 37°C. Dose 0 reflects aldosterone secretion into the medium stimulated with 5-HT 10^{-7}M. Aldosterone secretion into the medium was measured by RIA. Data from 4 separate experiments, with triplicate incubations performed in each, were combined and expressed as mean±SEM.

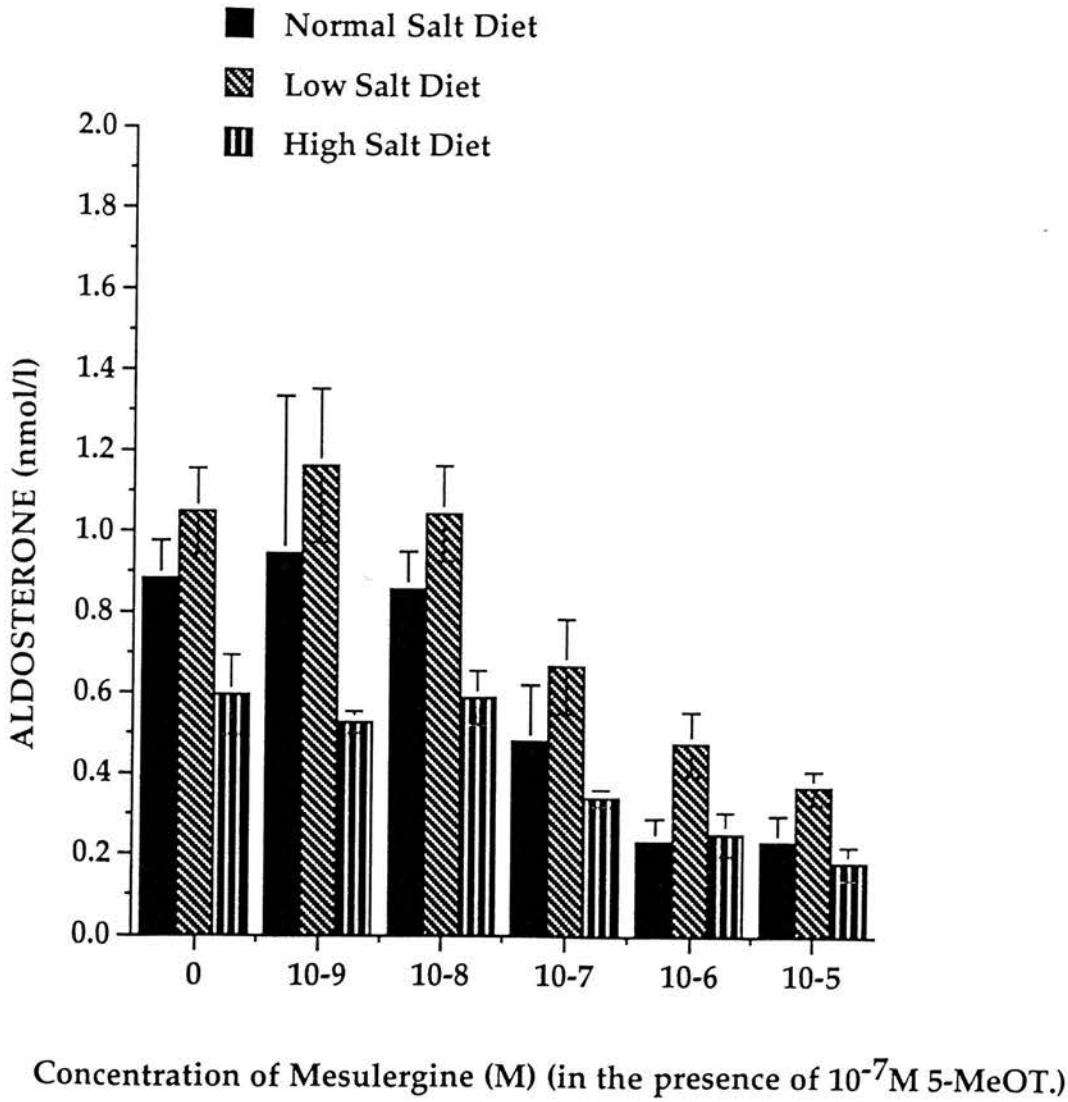


Figure 4.13. *Dose-dependent increase in aldosterone secretion from isolated ZG cells, prepared from rats fed on a normal, low and high salt diet, in response to increasing concentrations of mesulergine in the presence of 5-MeOT $10^{-7}M$.* Female Wistar rats were fed on either a normal (1%), low (0%) or high (3%) salt diet for one week prior to removal of their adrenal glands. Isolated rat ZG cells were then prepared and incubated with increasing concentrations of mesulergine in the presence of 5-MeOT 10^{-7}M for 1 hour at 37°C. Dose 0 reflects aldosterone secretion into the medium stimulated with 5-MeOT 10^{-7}M. Aldosterone secretion into the medium was measured by RIA. Data from 4 separate experiments, with triplicate incubations performed in each, were combined and expressed as mean±SEM.

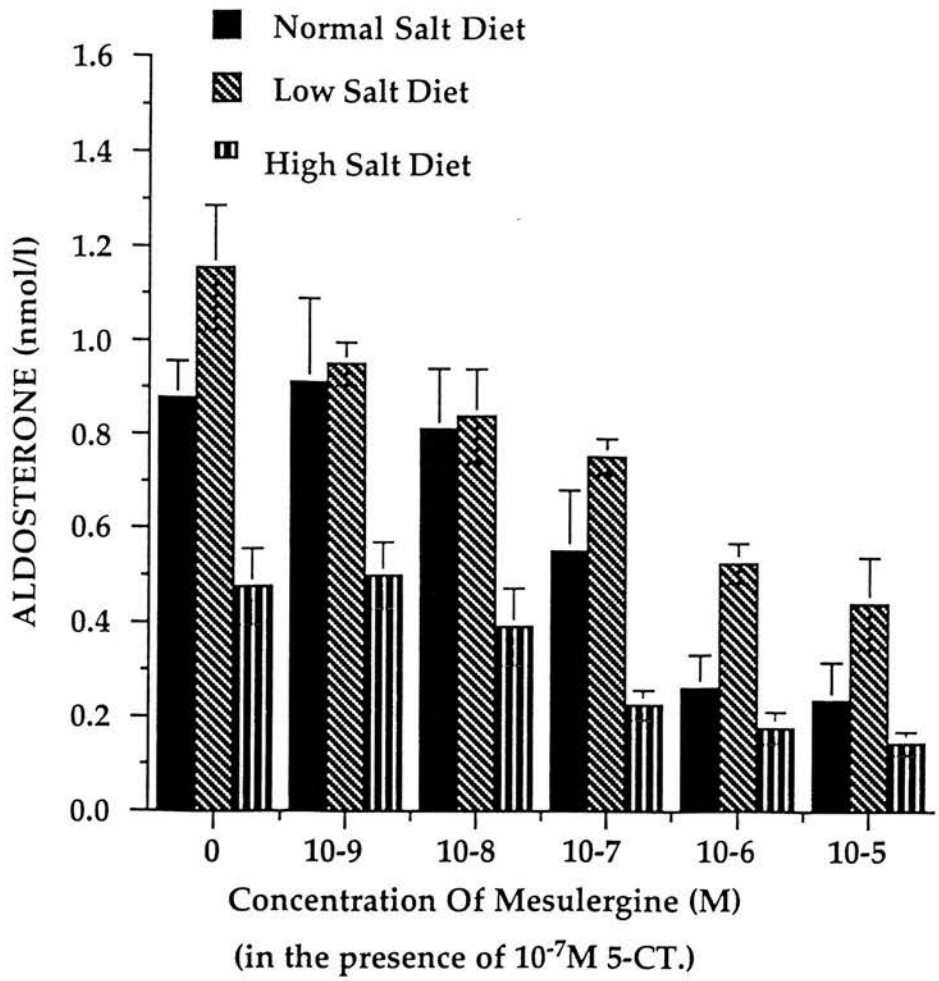


Figure 4.14. *Dose-dependent increase in aldosterone secretion from isolated ZG cells, prepared from rats fed on a normal, low and high salt diet, in response to increasing concentrations of mesulergine in the presence of 5-CT $10^{-7}M$.* Female Wistar rats were fed on either a normal (1%), low (0%) or high (3%) salt diet for one week prior to removal of their adrenal glands. Isolated rat ZG cells were then prepared and incubated with increasing concentrations of mesulergine in the presence of 5-CT 10^{-7}M for 1 hour at 37°C. Dose 0 reflects aldosterone secretion into the medium stimulated with 5-CT 10^{-7}M. Aldosterone secretion into the medium was measured by RIA. Data from 4 separate experiments, with triplicate incubations performed in each, were combined and expressed as mean±SEM.

inhibition of all three agonists in each experiment. Mesulergine itself did not produce any effect on aldosterone secretion (data not shown).

4.4. Discussion.

The effects of a number of 5-HT agonists and antagonists, on the secretion of aldosterone from the rat ZG were studied, in an attempt to characterise the 5-HT receptor present within the rat ZG. The lack of availability of a suitable radioactive derivative of 5-HT makes radioligand binding studies in adrenal cells difficult, ^{3}H-5-HT has a very low specific activity whilst ^{125}I-5-HT is unstable and causes structural distortion of the 5-HT molecule. These technical limitations necessitate the use of largely indirect pharmacological studies on the end steroid response to 5-HTergic stimulation. An ideal study would utilise a wide range of agonists and agonists to 5-HT to characterise the functional response of the cell type, allowing identification of the 5-HT receptor present. This would then be confirmed with the use of a selective 5-HT radioligand in binding studies.

5-HT agonists and antagonists have various degrees of specificity for specific 5-HT receptors, making a definitive characterisation of a 5-HT receptor with pharmacological studies alone difficult. For example, mesulergine is an antagonist at the 5-HT_2 and 5-HT_7 receptor sites, with very weak antagonistic effects at the 5-HT_1 and 5-HT_6 and 5-HT_5 sites. Ketanserin is a highly selective antagonist for the 5-HT_2 site, also showing weak antagonistic properties at the 5-HT_1, 5-HT_4 and 5-HT_7 sites. The compounds BIMU 1, BIMU 8, zacopride and cisapride, are all selective agonists at the 5-HT_4 site, however they also act as antagonists at the 5-HT_3 site. ICS 205/930, although selective for the 5-HT_3 receptor does also act as a weak antagonist at the 5-HT_4 site, and DAU 6285 also acts as an antagonist at the 5-HT_4 and the 5-HT_3 site. The indole compounds 5-methoxytryptamine and 5-carboxamidotryptamine are fairly

non-selective agonists, acting preferentially at the 5-HT_1, 5-HT_4, 5-HT_6 and 5-HT_7 receptor sites (Reviewed in Boess & Martin, 1993). A table summarising pKd values for the compounds tested in this study is shown (Table 4.4 & Table 4.5)(Reviewed in Boess & Martin, 1993).

The results obtained in this study confirm those of other groups that 5-HT acts directly on the ZG to increase aldosterone secretion *in vitro*, (Muller & Zeigler, 1968; Haning *et al.*, 1970; Tait *et al.*, 1972; Bing & Schulster, 1977). 5-HT caused a 4 to 6 fold increase in aldosterone output, reaching maximal stimulation at approximately 10^{-6}M. This compares favourably with a number of groups, but is slightly lower than the 8 fold increase observed by Tait *et al.*, (1972). Also a rise in cAMP was seen following stimulation of aldosterone secretion by 5-HT, which is in agreement with various studies that cAMP is the second messenger system for 5-HT induced aldosterone secretion from the ZG.

Six antagonists were used and their ability to inhibit the stimulatory capacity of 5-HT on aldosterone output was studied. Based purely on the % inhibition from the four combined experiments, the order of effectiveness of the antagonists was mesulergine>ketanserin>ICS 205/930>cyanopindolol>CV 205 502>DAU 6285, the latter two showing very slight but non significant inhibition (Table 4.2). Of the agonists used, 5-methoxytryptamine and 5-carboxamidotryptamine displayed aldosterone stimulating properties almost identical to that of 5-HT. BIMU 1, BIMU8, zacopride and cisapride displayed no aldosterone stimulating activity in the rat zona glomerulosa.

The antagonists ketanserin and mesulergine, not only inhibited the stimulation of aldosterone by 5-HT, but also an inhibition of basal aldosterone levels was also observed. This effect has been observed by other groups working with 5-HT

antagonists (Muller & Zeigler , 1968; Matsuoka *et al.*, 1985). This basal inhibition is most probably due to the presence of endogenous 5-HT within the rat adrenal gland, namely within the medulla and mast cells present throughout the adrenal cortex (Verhofstadt & Jonsson, 1983; Holzwarth *et al.*, 1984; Lefebvre *et al.*, 1992). This endogenous 5-HT may occupy 5-HT receptors present within the rat ZG, exerting a tonic stimulatory influence on aldosterone secretion, and this would be suppressed with the introduction of 5-HT antagonists to the cells. However, although radiolabelling studies have shown the rapid uptake of 5-HT and its metabolism to 5-HIAA, there is very little conclusive evidence of storage of 5-HT in capsular tissue (Trost & Muller, 1976). In the study detailed in Chapter 5, immunohistochemical staining of sections of the rat adrenal cortex exhibited no staining for 5-HT. One possibility is the presence of chromaffin cells within the adrenal cortex as described by Bornstein *et al.*, (1992), which contain 5-HT. It is also feasible that the concentration of 5-HT may be linked to stress levels within the rat preceding cervical dislocation. Stress facilitates the release of ACTH, resulting in increased adrenal perfusion by the blood. 5-HT which is transported by the platelets, may then be released locally, and bind to the receptor. Alternatively, the 5-HT antagonists may also exhibit partial dopaminergic agonist properties, which would enhance the tonic inhibitory influence of dopamine on aldosterone secretion, resulting in a decreased basal output (Carey *et al.*, 1979). High affinities for 5-HT antagonists for dopamine receptors has been reported by Leyson *et al.*, (1981), spiperone and haloperidol were 7 and 40 times more potent at dopamine than at 5-HT_2 receptors. A high affinity for the dopamine receptor was also reported for methysergide. However only very high doses of dopamine have been shown to inhibit basal aldosterone secretion ($>10^{-5}$M), and the effect seen was only moderate. Therefore it is unlikely that a lower dose of a 5-HT antagonist would act on dopamine receptors (Aguilera & Catt, 1984).

From the results in this study, the antagonists, ICS 205/930, DAU 6285 and CV 205 502 all produced slight inhibition of the 5-HT induced aldosterone secretion. This data taken together with the reported pKd values of these compounds and the second messenger systems linked to the receptors (Table 4.4), would negate the presence of 5-HT_{1a}, 5-HT_{1b}, 5-HT_3 and 5-HT_4 receptors, within the rat ZG. The data from the 5-HT_4 agonists used would also negate the presence of a 5-HT_4 receptor in the rat zona glomerulosa. As no selective antagonists for the 5-HT_{5a} or 5-HT_{5b} receptors were looked at the possibility of this receptor being present within the adrenal gland of the rat can not be ruled out. In fact the agonist 5-carboxamidotryptamine is active at this receptor also. The second messenger system has still to be identified for this receptor type.

Historically, the receptor present within the zona glomerulosa of the rat for 5-HT was thought to be of the formerly known '5-HT_{1c}/5-HT_2' type (i.e the 5-HT_{2c} site), although the second messenger for these receptors was the PI system, and not cAMP which is the second messenger for 5-HT induced aldosterone secretion. Certainly, the results with the antagonists ketanserin and mesulergine, would suggest the presence of a 5-HT receptor in the rat zona glomerulosa of this type. Mesulergine, however, acts also as an antagonist at the 5-HT_1 and 5-HT_7 receptor sites.

Studies in the frog interrenal gland and the human adrenal gland by Lefebvre *et al.*, (1992, 1993, 1996; Idres *et al.*, 1991), have provided conclusive evidence for the presence of a 5-HT_4 receptor present within the adrenal cortex of these species. Agonists BIMU 1 and BIMU 8, are members of the azabicycloalkyl benzimidazolones, they produced a comparable stimulation to 5-HT of aldosterone secretion from frog and human adrenal gland. BIMU 8 was more potent in this respect than BIMU 1 in these preparations. Also the agonists zacopride and cisapride in frog interrenal tissue

and the human adrenal gland, produced comparable stimulation of aldosterone form the zona glomerulosa to that produced by 5-HT, with the compound zacopride being more potent than cisapride (Lefebvre *et al.*, 1995).

The 5-HT_4 receptor was first named by Dumuis *et al.*, (1988), following their identification of a novel 5-HT receptor linked positively to adenylate cyclase in neonatal mouse collicular neurones. This receptor is a member of the G-protein coupled receptors, with cAMP acting as the principal second messenger. The receptor site shows significant similarities to the receptor subtype previously described by Shenker *et al.*, (1985, 1987), as a central 5-HT receptor positively coupled to adenylate cyclase, not classifiable as a 5-HT_1-like, 5-HT_2 or 5-HT_3 receptor. From various studies (Baxter *et al.*, 1991; Kaumann *et al.*, 1991), the bulk of evidence does not support receptor heterogeneity. It has been proposed that these differences may be due to the existence of different receptor subtypes (Eglen *et al.*, 1996), however the existence of 5-HT_4 receptor splice variants is a more likely explanation of tissular pharmacological differences. The receptor site was cloned in the rat in 1995 by Gerald *et al.*, who also characterized two splice variants 5-HT_{4a} and 5-HT_{4b}. Since then the number of splice variants is on the increase. Ullmer *et al.*, (1995), described the possible existence of splice variants existing in the second extracellular loop. Two novel splice variants in mouse have been reported by Claeysen *et al.*, (1998) 5-HT_{4c} and 5-HT_{4d}, and in human 5-HT_{4b} and 5-HT_{4e} and one in rat, 5-HT_{4c}. Pharmacologically these receptors are resistant to blockade by antagonists for the 5-HT_1-like, 5-HT_2 and 5-HT_3 receptors. Agonists at this receptor fall into three categories, the substituted benzamides, zacopride, cisapride, etc., the substituted benzimidazolones, BIMU 1, BIMU 8, etc. (Dumuis *et al.*, 1992) and indole derivatives 5-methoxytryptamine and 5-HT. Tryptamine acts as a low affinity partial agonist and 5-carboxamidotryptamine at very high concentrations will produce an effect as effective

as 5-HT. The compounds tropisetron, SDZ 205-557 and DAU 6285 act as antagonists at this receptor (Buchheit *et al.*, 1991; Dumuis *et al.*, 1992). These receptors have a wide distribution both within and across species (discussed in Chapter 1), in particular they are present within the human adrenal gland and frog interrenal gland (Lefebvre *et al.*, 1992, 1993).

The results from this study do not concur with that found in the frog and human adrenal glands. The 5-HT_4 antagonist used, DAU 6285, did not produce a significant inhibition of 5-HT stimulated aldosterone secretion, at doses up to 10^{-5}M. 5-HT_4 agonists from all three classes were used, the substituted benzamides zacopride and cisapride produced no significant stimulation of aldosterone secretion from isolated rat zona glomerulosa cells, the substituted benzimidazolones BIMU 1 and BIMU 8 again produced no significant stimulation of aldosterone secretion, the indole derivatives 5-HT and 5-methoxytryptamine on the other hand did produce significant stimulation of aldosterone secretion, however the indole 5-carboxamidotryptamine also produced strong aldosterone stimulation comparable to that of 5-HT, and this agonist at the 5-HT_4 site only displays aldosterone stimulating activity at much higher concentrations. Also these three agonists are also agonists at the 5-HT_6, 5-HT_1 and the 5-HT_7 receptor sites, suggesting the presence of a receptor site belonging to one of these families within the ZG. However the second messenger system present within the ZG would not support the existence of a 5-HT_1 site.

The 5-HT_6 receptor was cloned from a rat cDNA library, displays operational characteristics resembling a 5-HT_1 receptor and has nanomolar affinity for 5-carboxamidotryptamine and methiothepin (Monsma *et al.*, 1993; Ruat *et al.*, 1993a). The receptor is positively coupled to adenylate cyclase, however the pharmacological profile is very different from the 5-HT_4 receptor. It is widely distributed in the brain,

and in the periphery and has been shown to be present within the stomach of some species and in the adrenal gland of the hamster (Ruat *et al.*, 1993a). This receptor site appears to be an interesting target for the treatment of CNS disorders, with little likelihood of peripheral side effects (Sleight *et al.*, 1998). The selective antagonists Ro 04-6790 and Ro 63-0563, will allow verification of this, and other specific effects of this receptor site and possible clinical uses (Sleight *et al.*, 1998).

The 5-HT_7 receptor has been cloned from rat, mouse and guinea pig cDNA libraries (Ruat *et al.*, 1993b; Plassat *et al.*, 1993; Tsou *et al.*, 1994). Again this receptor is positively coupled to adenylate cyclase, and is distributed widely throughout the brain, the heart, the intestine and in the periphery in some species (Plassat *et al.*, 1993). It displays a unique pharmacological profile, consistent across species. Based on brain localization and pharmacological studies, it has been suggested that these receptors may play a role in the control of circadian rhythms and have potential therapeutic roles in depression and schizophrenia (Sleight *et al.*, 1995). 5-HT, 5-methoxytryptamine and 5-carboxamidotryptamine act as agonists and mesulergine, methiothepin, ketanserin and ritanserin act as antagonists (Hirst *et al.*, 1996). Interestingly, the pharmacology of this receptor resembles that of a previously characterised 5-HT receptor which mediates smooth muscle relaxation directly and is positively coupled to cAMP (Sumner *et al.*, 1989; Plassat *et al.*, 1993). Also of interest the 5-HT_7 antagonists mesulergine, methiothepin and ritanserin were shown to be strong antagonists for the 5-HT induced aldosterone secretion in isolated rat zona glomerulosa cells, with estimated pKd values of 7.63, 7.18 and 7.06 respectively (Lisa Bishop, Honours Project, 1989. Table 4.3). Methiothepin is also an antagonist at the 5-HT_6 receptor site. These pKd values correspond strongly with reported pKd values for the 5-HT_7 receptor site in the rat (Table 4.4). Thus from the pharmacological studies reported in this chapter, plus the data from Lisa Bishop's project, and Joyce Yau's preliminary data, the presence of a 5-

HT_7 receptor within rat adrenal cortex is likely. This conclusion is also in accordance with studies very recently reported by Contesse *et al.*, (1999).

The agonists 5-carboxamidotryptamine and 5-methoxytryptamine were also studied in animals placed on a low salt diet and animals on a high salt diet (Figure 4.12). Sodium status is the main regulator of aldosterone secretion. Zona glomerulosa cells prepared from animals on a low sodium diet or a high sodium diet, as expected have altered basal aldosterone levels, as a result of altered mineralocorticoid output from the adrenal cortex, in order to maintain physiologically acceptable electrolyte levels. All three agonists produced greater stimulation of aldosterone in the low sodium status as compared to the normal and high sodium status, and this was significantly different. This may be due to upregulation/downregulation of the receptors present within the zona glomerulosa of the rat, although further studies are needed before this can be ascertained. Preliminary studies utilising a 5-HT_7 probe in sections of rat adrenal gland by Dr. Joyce Yau (WGH), would suggest the existence of a 5-HT_7 receptor, and sections taken from animals on either a low sodium diet or a high sodium diet if probed with this would indicate if the receptors present are regulated in any way.

If 5-HT plays a physiological role in the control of aldosterone secretion, the amine must be secreted locally or be present within the gland to act as a secretagogue. 5-HT circulates free in plasma in very low concentrations as the majority of the amine is sequestered and stored within platelets. 5-HT is converted from the precursor 5-HTP by the enzyme, L-aromatic amino acid decarboxylase (L-AAAD). In vitro studies indicate that the adrenal cortex has the ability to synthesise 5-HT from its precursor (Delarue *et al.*, 1992), suggesting L-AAAD is present in the adrenal gland. This has been further supported by immunohistochemical localisation of L-AAAD within the cortex (Sephton, 1995). This will be discussed in more detail in Chapter 5.

The pharmacological profile and putative identification of a second messenger system suggests the existence of specific 5-HT receptors within the rat zona glomerulosa. However conclusive evidence requires a more detailed pharmacological profile and molecular experiments, probing the adrenal cortex for specific amino acid sequences encoding a specific 5-HT receptor. Preliminary studies in rat adrenal sections with the 5-HT_7 probe, have picked up the existence of a 5-HT_7 receptor site (Joyce Yau, WGH). This taken together with the pharmacological studies detailed above would suggest the existence of a 5-HT receptor site in the rat zona glomerulosa of the 5-HT_7 class, although the presence of another 5-HT receptor can not be ruled out. In particular the antagonists ketanserin and cyanopindolol, both produced significant inhibition of the aldosterone response at higher concentrations. Further experiments such as concomitant incubations with 5-HT and agonists for the 5-HT_2 and 5-HT_1 receptor families, plus incubations with 5-CT and 5-MeOT with antagonists for these receptor sites, will allow further definition of the receptor sites present within the rat adrenal cortex. The presence of the 5-HT_7 receptor in the rat ZG was reported by Contesse *et* al., (1999). Interestingly, western blotting in the inner zones of the rat adrenal cortex by Contesse *et al.*, (1999), found that the 5-HT_7 receptor site was expressed by the ZG/ZF and the ZR. The actions of 5-HT and a variety of agonists and antagonists should be looked at within this area of the rat adrenal gland. With the advent of new and more selective antagonist radioligands, such as [^{3}H]-SB-2669970 (Thomas *et al.*, 2000), this receptor site may be more fully characterised, and the regulation of these receptors within the adrenal cortex may be more fully investigated.

Molecular genetics is providing novel insights into 5-HT receptor function, which may allow for a clearer understanding of 5-HT receptors in human disease. For example, usage of inducible knockout strategies to probe the functions of 5-HT receptors has

provided animal models for studying underlying aggressive behaviour and responses to drug abuse, as in the case of the 5-HT_{1b} knockout mouse (Rocha *et al.*, 1997). Clinical development of new, more selective antagonists and ligands will allow evaluation of the importance of 5-HT in the adrenal gland. Pharmacological blockade of the receptor site within the ZG will allow new perspectives for the treatment of various aldosterone disorders such as primary hypoaldosteronism.

Table 4.1.

Properties of the 5-HT Agonists and Antagonists.

Agonist or Antagonist	Molecular Weight	Solubility	Receptor Specificity
Mesulergine	362.5	Saline	5-$HT_{2/7}$ Antagonist
Ketanserin	545.5	Saline	5-HT_2 Antagonist
ICS 205/930	320.8	Saline	5-HT_3 / 5-HT_4 Non-selective Antagonist
Cyanopindolol	287.3	EtOH	5-$HT_{1a/1b}$ Antagonist
Zacopride	364.28	Saline	5-HT_4 Agonist / 5-HT_3 Antagonist
Cisapride	483.97	Saline	5-HT_4 Agonist / 5-HT_3 Antagonist
DAU 6285	367.87	Saline	5-HT_3 / 5-HT_4 Non-selective Antagonist
BIMU 1	382.89	Saline	5-HT_4 Agonist / 5-HT_3 Antagonist
BIMU 8	378.9	Saline	5-HT_4 Agonist / 5-HT_3 Antagonist
5-MeOtryptamine	190.2	Saline	5-$HT_{4/6/7}$ Agonist
5-Carboxamidotrytamine	319.32	Saline	5-$HT_{1/5/6/7}$ Agonist
CV 205 502	432.03	Saline	5-HT_3 Antagonist

Table 4.2

% Inhibition ± SEM of the antagonists tested (at concentration 10^{-6}M).

Compound	% Inhibition ± SEM
Ketanserin	**81.3% ± 2%**
Mesulergine	**86.6% ± 2.6%**
DAU 6285	**13.8% ± 3%**
CV 205 502	**34.5% ± 4%**
ICS 205-930	**36.2% ± 3.7%**
Cyanopindolol	**65.6% ± 5%**

Table 4.3

The estimated pK_D values of 5-HT antagonists for the rat zona glomerulosa receptor.

Drug	pK_D
Ketanserin	**6.01**
Mesulergine	**7.63**
Cyanopindolol	**5.73**
ICS 205 930	**5.4**

(Taken from Lisa Bishop's honours project)

Table 4.4

pKd Values for the antagonists tested (Adapted from Boess & Martin, 1993).

Compound	5-HT_{1A}	5-HT_{1B}	5-HT_{1D}	5-HT_{1E}	5-HT_{1F}	5-HT_{2A}	5-HT_{2B}
Ketanserin	**5.86**	**5.72**	**6**	**< 6**	**< 6**	**8.7**	**5.45**
DAU 6285	-	-	-	-	-	-	-
Mesulergine	**6.23**	**4.88**	**5.2**	**< 6**	**< 6**	**6.89**	**7.44**
ICS 205930	**4.7**	**4.35**	-	-	-	**5.28**	-
Cyanopindolol	**8.27**	**8.28**	**6.85**	**< 6**	**< 6**	**4.53**	-
CV 205 502							

Compound	5-HT_{2C}	5-HT_3	5-HT_4	5-HT_{5a}	5-HT_{5b}	5-HT_6	5-HT_7
Ketanserin	**7.32**	-	**< 5**	**< 6**	**< 6**	-	**App. 6-7**
DAU 6285	-	-	**7.8**	-	-	**App. 6.6-6.1**	**App. 6-7**
Mesulergine	**8.52**	-	-	**< 6**	**< 6**	**App. 6.6-6.1**	**7.68**
ICS 205930	**4.59**	**7.6**	-	-	-	-	-
Cyanopindolol	**4.44**	-	-	-	-	-	**App. 6-7**
CV 205 502							

Table 4.5

PKd values for the agonists tested (Adapted from Boess & Martin, 1993).

Compound	5-HT_{1A}	5-HT_{1B}	5-HT_{1D}	5-HT_{1E}	5-HT_{1F}	5-HT_{2A}	5-HT_{2B}
BIMU 1	-	-	-	-	-	-	-
BIMU 8	-	-	-	-	-	-	-
Zacopride	-	-	-	-	-	-	-
Cisapride	-	-	-	-	-	-	-
5-CT	**App. 9**	**8.14**	**9.43**	**< 6**	**< 6**	-	**6.82**
5-MeOT	-	**7.34**	**8.62**	**< 6**	**< 6**	-	**8.04**
Compound	5-HT_{2C}	5-HT_3	5-HT_4	5-HT_{5a}	5-HT_{5b}	5-HT_6	5-HT_7
BIMU 1	-	-	**7.9**	-	-	**< 6**	**6-7**
BIMU 8	-	-	**7.8**	-	-	-	**6-7**
Zacopride	-	-	**6.8**	-	-	**< 6**	**6-7**
Cisapride	-	-	**7.5**	-	-	-	-
5-CT	-	-	**5.2**	**6.4**	**6.63**	**< 6**	**9.8**
5-MeOT	-	-	**6.5**	-	-	**7.41**	**9.24**

Chapter Five

Role of L-Aromatic Amino Acid Decarboxylase in the Regulation of Aldosterone Secretion by 5-HT and Dopamine in the Rat Adrenal Gland.

5.1 Introduction.

In 1954, a link between sodium status and aldosterone secretion was first reported (Leutscher & Axelrad). Since that initial observation many studies have investigated the effect of sodium loading and/or sodium depletion on aldosterone secretion from the adrenal cortex. In fact sodium depletion is one of the most potent stimulators of aldosterone secretion in man and animals, also causing morphological changes within the ZG (Davis, 1967). The exact mechanism of action remains unclear due to the large amount of physiological effects initiated by altered sodium status. Also altered sodium status affects the aldosterone response to other regulators such as ACTH, AII and potassium (Muller, 1968, 1969 (Refer to Chapter 1)).

Both the biogenic amines 5-HT and dopamine have been shown in numerous studies to modulate the secretion of aldosterone from the ZG, in a range of species. It is not clear however if 5-HT, and /or dopamine, could have a role in the regulation of mineralocorticoid secretion physiologically. In particular, the physiological source of both these amines, reaching the ZG, is still under investigation.

Dopamine and 5-HT are present within the adrenal gland, 5-HT is present within the medulla and dopamine most probably within the ZG (McCarty *et al.*, 1986). Both regulators have receptor sites present within the ZG. Dopamine binding sites within the adrenal cortex have been identified, from autoradiographic experiments, as receptors of the D2 family within the ZG (Amenta *et al.*, 1994). Sibley & Monsma, 1992, further characterised these receptors as the D_4 type, negatively coupled to adenylyl cyclase. 5-HT receptors within the human adrenal gland have been identified as the 5-HT_7 type (Contesse *et al.*, 1999).

The concentration of free 5-HT in plasma (1-50nM) is within the range shown to directly stimulate aldosterone production *in vitro*, although too low a concentration to have any physiological effect *in vivo* also, free dopamine concentrations within the plasma appear to be too low to reach the high concentrations required to elicit changes in aldosterone secretion (Zinner *et al.*, 1983). This suggests a local delivery or production of these regulators to enable a physiological modulatory role in aldosterone production, either alone or by interacting with other regulators.

Both 5-HT and dopamine are converted from their precursors, 5-HTP and L-DOPA, respectively, by the enzyme L-AAAD. Plasma concentrations of 5-HTP and L-DOPA are sufficiently high to support the hypothesis that local production of these amines, by L-AAAD, could act as a paracrine mechanism for modulating aldosterone secretion. Immunohistochemistry investigations with an antibody to L-AAAD have shown the presence of the enzyme within the adrenal gland of several species (Rahman *et al.*, 1981). *In vitro* studies indicate that the adrenal gland has the ability to synthesise 5-HT from 5-HTP (Verhofstad & Jonsson, 1983; Delarue, 1992). In addition, Beltramo *et al.*, (1993), reported that biochemically L-AAAD activity was particularly high within the adrenal gland as compared to the brain, where its concentration is five fold lower. The availability of antibodies to L-AAAD has permitted direct visualisation of L-AAAD. Several groups using immunohistochemical techniques have shown the occurrence of L-AAAD in the adrenal gland. Beltramo *et al.*, (1993), reported that in the mouse adrenal gland, strong L-AAAD immunoreactive cells were found to be concentrated in the medulla, while a faint diffuse immunoreactivity was present in the ZF. Within the hamster adrenal gland L-AAAD immunoreactivity was found present in

the ZF alone (Lugo *et* al., 1988). No study has demonstrated the presence of L-AAAD immunoreactivity within the ZG of any species.

The presence of L-3,4-dihydroxyphenylalanine (L-DOPA) decarboxylase, was first reported in mammalian kidney extracts (Holtz *et al.*, 1938). A role for this enzyme in the synthesis of catecholamines in mammalian tissues was then postulated by Blaschko *et al.*, (1939). Subsequently it was reported that 5-HTP was readily decarboxylated by extracts of mammalian kidney and liver, and a role for a 5-HTP decarboxylase enzyme in the synthesis of 5-HT was postulated (Udenfriend *et al.*, 1953; Clark *et al.*, 1954). In 1962 the name aromatic L-amino acid decarboxylase (L-AAAD) was proposed as the enzyme was found to have affinity for a broad number of substrates, catalyzing the decarboxylation of a number of aromatic L-amino acids and their α-methylated derivatives (Lovenberg *et al.*, 1962).

L-AAAD catalyzes the formation of dopamine from L-DOPA, 5-HT from 5-HTP, tyramine from tyrosine, 2-phenylethylamine from phenylalanine and tryptamine from tryptophan. In addition L-AAAD may have as yet unidentified functions in nonmonoamine neurons in which L-AAAD has been localized (Reviewed by Zhu & Juorio, 1994).

L-AAAD was first purified from hog kidney (Christenson *et al.*, 1970) and subsequently from rat liver (Ando-Yamamoto *et al.*, 1987), rat kidney (Shirota & Fujisawa, 1988), bovine brain (Nishigaki *et al.*, 1988), rat pheochromocytoma (Coge *et al.*, 1990) and human pheochromocytoma (Maneckjee & Baylin, 1983; Shirota &

Fujisawa, 1988) cells. The primary structures are remarkably conserved between species. The molecular weight of brain and adrenal medulla L-AAAD is around 100kDa (85-112 kDa) (Christenson *et al.*, 1970; Lancaster & Sourkes, 1972). Initially the molecule was thought to consist of three subunits, however most studies now concur that L-AAAD consists of two subunits (Lancaster & Sourkes. 1972; Ichinose *et al.*, 1985). Pyridoxal phosphate (PLP), acts as a co-factor for L-AAAD (Rahman *et al.*, 1982). The molecule is tightly bound to the ε-amino group of a lysine residue of L-AAAD (Bossa *et al.*, 1977). The enzyme is not saturated *in vivo* (Bowsher & Henry, 1986), and thus is not the rate-limiting step in the synthesis of dopamine and 5-HT.

The enzyme is widely distributed within neural tissues where it plays a neuron-specific role as a neurotransmitter biosynthetic enzyme, and in extra-neuronal tissues where it acts as a nonspecific decarboxylating enzyme, and perhaps other so far undetermined functions. L-AAAD is mainly localized in catecholamine and 5-HT containing neurons in the CNS (Hokfelt *et al.*, 1973). Namely, cerebral cortex, brainstem, hypothalamus, caudate nucleus, hippocampus and cerebellum in a variety of species to varying degrees (Rahman *et al.*, 1981). Peripherally, L-AAAD has been located in the kidney, liver, pancreas and adrenal gland (Rahman *et al.*, 1981). In the kidney L-AAAD is localised in the proximal convoluted tubules, and straight tubules, and not associated with nerves. L-AAAD presence within the kidney and liver is surprising as biosynthesis of monoamines does not appear to occur in these tissues. The physiological significance of the presence of L-AAAD in these tissues is not yet clear, but perhaps the enzyme may have a fundamental role in the metabolism of other aromatic amino acids. Extraneuronal dopamine is thought to be synthesized by L-AAAD in the proximal tubular cells (Baines *et al.*, 1985). Baines *et al* (1985) also demonstrated that L-DOPA was converted to dopamine by a proximal convoluted tubule-enriched fraction from rat kidneys. Also, Rahman *et al.*, (1981), demonstrated, using L-DOPA and 5-HTP as

substrates, that the serum of monkeys, guinea pigs and rats contain L-AAAD activity. Immunohistochemistry techniques have identified L-AAAD containing cells in the tracheobronchial epithelium, neuroepithelial bodies in the bronchopulmonary epithelium, Kultschitzky cells in the small intestine and appendix as well as in the adrenal chromaffin cells. The aforementioned cells all belong to the amine precursor uptake decarboxylation (APUD) system (Pearse, 1974). The existence of L-AAAD gene expression within glial cells was demonstrated in 1992 (Li *et al.*, 1992). In normal striatum L-AAAD content of glial cells is unlikely to contribute to catecholamine production. However it is possible that some trace amines such as tyramines or tryptamine could be formed by L-AAAD in glial cells. These trace amines in turn could then modulate monoaminergic neurotransmission (Paterson *et al.*, 1990). L-AAAD activity in glial cells may be an additional area where decarboxylation of L-DOPA may occur, an area important in Parkinsonian patients where most of the striatel dopamine terminals have been lost and exogenous L-DOPA may be decarboxylated by glial L-AAAD. In fact L-AAAD may play an important role in Parkinson's disease (Dairman *et al.*, 1972). L-DOPA is an effective treatment in this disease state.

For a period of time it was thought that there was a family of L-AAAD enzymes. Based on a number of observations, it was suggested that different enzymes catalyze the decarboxylation of L-DOPA and 5-HTP. Pharmacological studies on the decarboxylation of 5-HTP and L-DOPA in rat brain concluded that different optimal temperatures, pH and substrate concentration were required (Slow & Dakshinamurti, 1985). Another observation was that if only one L-AAAD enzyme existed, then the ratio of the enzyme activities with regard to L-DOPA and 5-HTP should be closely similar in all tissues. However this has not always been shown to be the case. Furthermore, in brain 5-HTP decarboxylase is equally distributed between soluble and

particulate fractions in contrast to L-DOPA decarboxylase, which is mainly soluble (Sims *et al.*, 1973). This dissimilar subcellular distribution has supported the existence of L-AAAD isozymes in the brain (Rahman & Nagatsu, 1982). Also, within hypothalamic nuclei, the distribution of DOPA decarboxylase correlates directly with those of NA and DOPA, but not with that of 5-HTP decarboxylase (Palkovits *et al.*, 1974).

Bender & Coulson (1972), first suggested the existence of one L-AAAD enzyme, with a single catalytic site, containing different binding sites for the two substrates, based on their studies of the kinetics of the decarboxylases. This hypotheses has been supported by numerous studies, including those of the investigation of the nucleotide sequences coding for the synthesis of L-AAAD. In a study by Zuo & Yu, (1991), the L-DOPA/5-HTP decarboxylation ratios were not too dissimilar in a variety of tissues including cat brain and rat brain. The isolated enzymes from pig kidney, rat kidney, guinea pig kidney, human phaeochromocytoma and bovine brain can catalyze the decarboxylation of both L-DOPA and 5-HTP (Nishigaki *et al.*, 1988). Immunological studies support the existence of one single enzyme. The enzyme present within the adrenal medulla, pineal gland, liver, kidney and striatum is identical with respect to immunological crossreactivity and molecular size (Shirota & Fujisawa, 1988). Antiserum prepared against purified L-AAAD from bovine adrenal or rat kidney, both inhibit L-DOPA and 5-HTP decarboxylase (Shiroto & Fujisawa, 1988).

Molecular biological investigations have provided the most convincing evidence for the presence of a single L-AAAD enzyme. The presence of a single mRNA species in bovine liver, kidney and adrenal as well as rat liver, brain and phaochromocytoma cells and human phaochromocytoma cells has been shown utilising hybridization analysis with a cDNA probe complementary to bovine adrenal L-AAAD mRNA (Albert *et al.*,

1987). Tison *et al* (1991), described L-AAAD gene expression in dopaminergic and 5-HTergic neurones of rat brain, utilizing *in situ* hybridization. Definitive proof of a single enzyme was provided by Sumi *et al*, (1990), who demonstrated that the recombinant human L-AAAD decarboxylates 5-HTP to 5-HT as well as L-DOPA to dopamine. Further to this study analysis with probes corresponding to the entire coding region of L-AAAD pheochromocytoma cDNA, the L-AAAD primary sequence in both neuronal and non-neuronal tissue has been shown to be identical (Coge *et al.*, 1990). Following microinjection into *xenopus laevis* oocytes mRNA coding for bovine adrenal medulla L-AAAD has been expressed. This expressed enzyme activity was stereoselective for L-5-HTP and L-DOPA and blocked by NSD-1015. Also heating the expressed enzyme at 55°C produced a parallel loss of activity towards both substrates (Gudehithlu *et al.*, 1992). The aforementioned studies provide definitive proof that the enzymatic decarboxylation of both L-DOPA and 5-HTP is processed by a protein issued from the same gene and the same mRNA.

There are however, species and specific tissue variants of L-AAAD. The study of L-AAAD from the insect *Drosophila* demonstrated that different forms of the enzyme were produced by different mRNAs, depending on the tissue examined (Morgan *et al.*, 1986). Krieger *et al.*, (1991) found the presence of two species of L-AAAD mRNA in rat pheochromocytoma differing in their 5' untranslated region, one specific for neuronal tissue, the other non-neuronal. This may be due to a unique gene encoding for DOPA decarboxylase, giving rise to at least two transcripts, in response to differing signals during development.

Further to the studies mentioned above the aim of this study was to firstly localise the enzyme L-AAAD within rat adrenal gland utilising specific monoclonal antibodies against the purified enzyme, also to determine if 5-HT itself is localised within the rat

adrenal gland, (in association with specific 5-HT containing nerves), utilising a specific monoclonal antibody to 5-HT. Secondly to determine whether the rat adrenal ZG can synthesize 5-HT from 5-HTP or L-tryptophan, and dopamine from L-DOPA. Thirdly, to determine if sodium status, a well established modulator of aldosterone, can modulate the activity of L-AAAD in the rat ZG. Also to determine if 5-HTP or L-tryptophan are able to stimulate aldosterone secretion from whole rat adrenal capsules and isolated ZG cells, and whether L-DOPA can inhibit aldosterone secretion from whole adrenal capsular tissue and isolated ZG cells.

5.2. Data Analysis

Results are shown as mean + S.E.M. *n* refers to the number of experiments performed. Significance between basal and test results, from a particular salt diet, was assessed by Student's t-test. The significance between salt diets was assessed by analysis of variance. *P<0.05, **P<0.01 and ***P<0.001 were considered significant.

5.3 Results

An initial set of experiments carried out using rat adrenal capsules with L-tryptophan (10^{-6}M-10^{-3}M) as substrate demonstrated that this precursor to 5-HT did not stimulate aldosterone secretion. Neither was L-tryptophan converted to 5-HTP or 5-HT by rat adrenal capsules (data not shown).

Figure 5.1 shows the dose-response relationship between the concentration of 5-HTP and the biosynthesis of aldosterone from 4 different experiments using adrenal cortex capsules (this includes the ZG but not the ZF), obtained from rats on a normal salt diet.

Basal aldosterone secretion was 20.5nmoles/mg of protein ± 1.2 and significant stimulation of aldosterone occurred at a concentration of $5x10^{-5}$M 5-HTP ($p<0.05$). The maximal aldosterone response was observed at 10^{-4}M 5-HTP ($p<0.01$). In the presence of carbidopa, a specific inhibitor of L-AAAD, the aldosterone response to 5-HTP (10^{-4}M) was completely inhibited.

Figure 5.2 shows the aldosterone dose response to 5-HTP, in the absence and presence of carbidopa, from 4 experiments carried out using whole adrenal capsules obtained from rats maintained on a normal, low and high salt diet. The basal aldosterone secretion in capsules obtained from rats on a normal salt diet was 27.7 nmoles/mg of protein ±2.7, and this increased to 33.2 nmoles/mg of protein ± 3.7 in low salt diet rats and decreased to 19.7 nmoles/mg of protein ± 2.5 in high salt diet rats which was a significant decrease. In the normal salt diet group, the minimal dose of 5-HTP to cause a significant aldosterone secretion was 10^{-4}M . In low and high salt diet groups, the minimal dose of 5-HTP which caused a significant ($p<0.05$) effect was $5x10^{-5}$M. The maximal aldosterone secretion was 84.4 nmoles/mg of protein ± 5.10 in low salt diet as compared to 43.1 nmoles/mg of protein ± 1.7 in normal salt diet rats ($p<0.05$). In all salt diet groups, aldosterone secretion in the presence of carbidopa, and 5-HTP, was not significantly different from basal secretion levels. Analysis of variance for the complete dose-response curves to 5-HTP showed that all diets were significantly different from each other ($p<0.05$). Thus, as expected, compared to the normal salt diet group, more aldosterone was produced in response to 5-HTP by the low salt diet group, while less was produced by rats from the high salt diet group.

Figure 5.3 illustrates the aldosterone dose-response to 5-HTP seen with isolated ZG cells obtained from rats on all three salt diet regimes (n=4). The basal aldosterone secretion in the normal salt diet group was 1.1 nmoles/mg of protein ± 0.07 and this increased to 2.0 nmoles/mg of protein ± 0.1 in the low salt diet group, and decreased to 0.5 nmoles/mg of protein ± 0.06 in the high salt diet group (not significant for n=4). In both the normal and high salt diet groups, a significant difference in aldosterone secretion as compared to basal levels was observed at 5-HTP incubations of 10^{-5}M to 10^{-3}M 5-HTP ($p<0.01$). In low salt diet animals, 10^{-6}M to 10^{-3}M 5-HTP produced a significant change in aldosterone secretion as compared to basal ($p<0.01$). Carbidopa (10^{-4}M) did not effect basal secretion of aldosterone, but did significantly inhibit the response to 10^{-4}M 5-HTP.

Plasma aldosterone concentrations from rats on normal, low and high salt diets are depicted in Figure 5.4a. Normal plasma levels were 1.1 nM/L±0.14. As expected this increased to 3.1nM/L±0.3 in the low salt diet group, and in the high salt diet group plasma levels were lower with a value of 0.341nM/L±0.09. The aldosterone plasma levels in each of the salt diets were significantly different to one another (Student's *t*-test, $p<0.001$). Figure 5.4b depicts the PRA values from the three different salt diets. As expected, the low salt diet regime caused a marked increase in PRA levels measured ($p<0.01$).

Figure 5.5a & 5.5b show the concentration of 5-HTP in rat adrenal capsules (n=4) and in renal cortex portions respectively, in tissues obtained from rats on normal, low and high salt diets, after incubation with increasing concentrations of 5-HTP, in the presence and absence of carbidopa, measured by HPLC. Also shown are the levels of 5-HTP at the end of the incubations with 5-HTP, the substrate for the L-AAAD enzyme. Since L-AAAD is known to be present within the renal cortex these incubations served as a positive control for the adrenal tissue incubations. The basal concentrations of 5-HTP were not significantly different from zero for adrenal capsules obtained from rats on all three salt diet regimes ($p<0.05$).. Carbidopa did not significantly affect the basal levels of 5-HTP or the levels of 5-HTP remaining at the end of the incubations.

The concentration of 5-HT when incubated with various concentrations of 5-HTP in the absence and presence of carbidopa (10^{-4}M) is shown in Figure 5.6a for the adrenal capsules and Figure 5.6b for the renal cortex tissue. In the adrenal capsule, for all three salt diets, the production of 5-HT increased dose-dependently, when incubated with concentrations of 5-HTP ranging from 10^{-6}M to $5x10^{-4}$M (n=4), with maximal 5-HT produced at a dose of $5x10^{-4}$M. The concentration of 5-HT observed in the low salt diet was significantly higher as compared to both normal and high salt diet tissue ($p<0.05$,n=4). In the presence of carbidopa, 5-HT production from 5-HTP appeared to be inhibited. In the kidney cortex, 5-HT also showed a dose-dependent increase when incubated with 5-HTP, while carbidopa also inhibited the production of 5-HT. No increase in aldosterone secretion with the low salt diet as compared to the normal salt diet, was observed with the kidney cortex tissue, as may have been expected. The

tissue was cut into sections of approximately the same size, not measured accurately, which may have affected the results.

Figure 5.7 shows the effect of increasing concentrations of 5-HTP and in the presence of carbidopa, on the production of 5-HIAA in a) adrenal capsule and b) kidney cortex. In both tissues the concentration of 5-HIAA produced does not appear to be affected by incubations with 5-HTP. There may have been a problem with the HPLC assay as the standard errors are very high. An increase in 5-HIAA measurement in urine, is often taken to indicate an increase in circulating plasma 5-HT, as in the carcinoid syndrome, however this is not the only metabolic pathway of 5-HT.

The blood levels of 5-HTP, 5-HT and 5-HIAA obtained from rats on all three salt diet regimes are illustrated in Figure 5.8a, and appear to not be modulated by salt intake. The results for 5-HT, 5-HTP and 5-HIAA in the urine were not significantly different between salt diets either (data not shown).

The next set of figures illustrates the dopamine side of this study. Figure 5.9 shows the mean concentrations of dopamine present in the urine obtained from rats on each of the salt diets. No significant difference existed between any of the values suggesting that sodium status has no effect on dopamine excretion. We did expect an increased dopamine output from animals on a high salt diet. Urinary dopamine values are difficult to measure due to oxidation, the urines were collected 12 hourly and maintained at pH3. However, the volume of urine changed from day to day, samples

sometimes were knocked over and food etc. ended up in the urine samples as well, making reproducible results difficult.

Figure 5.10 shows the dose response relationship between the concentration of L-DOPA and the biosynthesis of aldosterone from four experiments, using intact adrenal capsular tissue, from rats on a normal salt diet. Capsules were stimulated with 10^{-6}M 5-HT. Significant inhibition ($p<0.05$) was seen with L-DOPA concentrations above $5x10^{-5}$M. With the addition of carbidopa aldosterone secretion returned to near basal levels. The maximum inhibition was found with 10^{-4}M L-DOPA, which reduced aldosterone secretion from 19.1nmol/l (basal) to 10.1 nmol/l.

The aldosterone dose responses to 5-HT in the presence of increasing doses of L-DOPA from four experiments using adrenal capsules taken from rats on normal, low and high salt diets are depicted in Figure 5.11. The effect of carbidopa was also recorded. Basal secretion levels for normal, low and high salt diet groups were 18.1±3.086, 24.1±2.551 and 16.4±2.321 nmol/l respectively (not significantly different, n=4). L-DOPA administration appeared to reduce aldosterone biosynthesis at all doses, with inhibition increasing with increasing doses. The minimal dose required to cause a significant change in all three diet groups was $5x10^{-4}$M ($p<0.05$ (Normal & Low), $p<0.001$(High)). However significant changes were found with lower concentrations ($5x10^{-5}$M & 10^{-4}M) in the high salt diet group. The presence of carbidopa prevented any effect of L-DOPA. Aldosterone levels from these incubations were approximately the same as basal levels. In all cases, low salt diet groups had a

higher aldosterone output then normal salt diet groups, which in turn had higher levels than those in the high salt diet groups.

Figure 5.12 shows these changes in aldosterone secretion as a percentage change from basal levels. The maximum percentage inhibition for each group was 50.5% (normal), 32.7% (low) and 76.4% (high).

The aldosterone dose response relationship from incubations with isolated ZG cells are shown in Figure 5.13. In all incubations the presence of L-DOPA reduced aldosterone secretion from basal levels, 1.2±0.425 (normal), 1.5±0.259 (low) and 0.9±0.568 (high). Significant changes ($p<0.05$) were only seen in the low salt group using doses of L-DOPA above 10^{-4}M. The maximum inhibition found within each group was 58.95% (normal), 71.76% (low) and 78.74% (high).

The effect of L-DOPA administration on corticosterone biosynthesis by portions of inner zones of the adrenal cortex (containing ZF cells) and isolated ZF cells respectively was measured. No significant changes were seen in the whole portions of inner zone for any salt diet (n=4). In isolated cells, inhibition of secretion was seen at concentrations of L-DOPA above $5x10^{-4}$M in all salt diet groups, this was not significant (Figure 5.14).

Figure 5.15 illustrates the conversion of L-DOPA to dopamine by L-AAAD in both intact capsules and medullary portions taken from rats on normal salt diets, measured by HPLC. Due to technical problems analysis of samples on low and high salt diets

could not be undertaken. In both situations the amount of dopamine present within the sample increased dose-dependently with the increasing L-DOPA concentration, with concurrent carbidopa incubation reducing the production of dopamine.

Sections of kidney were used to perform control tests for staining with the L-AAAD monoclonal antibody Figure 5.16 shows a section through the kidney stained with (a)L-AAAD. Magnification was x 200. The L-AAAD immunoreactivity was observed throughout the kidney tubule, no L-AAAD immunoreactivity was evident within the glomeruli. Figure 5.17a) illustrates the general morphology of the rat adrenal gland through H/E staining at magnification of x80. The zonation of the gland can clearly be seen. Figure 5.17b) illustrates similar staining of a section of rat adrenal cortex at magnification x500, where differences in cell morphology can be observed. Immunohistochemical localisation of L-AAAD, using a specific monoclonal antibody raised against the purified enzyme, within the whole rat adrenal gland is shown in Figure 5.18a) (magnification x80), L-AAAD immunoreactive cells can be seen throughout the ZG, ZF and the adrenal medulla, no staining was evident within the adrenal capsule or the ZR. Figure 5.18b) illustrates the localisation of L-AAAD across a section of adrenal cortex (magnification x200). The presence of L-AAAD can be visualised within the medulla and cortex. Staining is apparent within the ZG and ZF. Figure 5.19a) illustrates immunoreactivity to 5-HT within the rat gut (control tissue sample), (b) illustrates the staining evident within the rat adrenal gland (magnification x 500), and it is evident that there is no 5-HT immunoreactive cells present within the adrenal capsule or ZG.

Graphical Illustration

- Chapter Five -

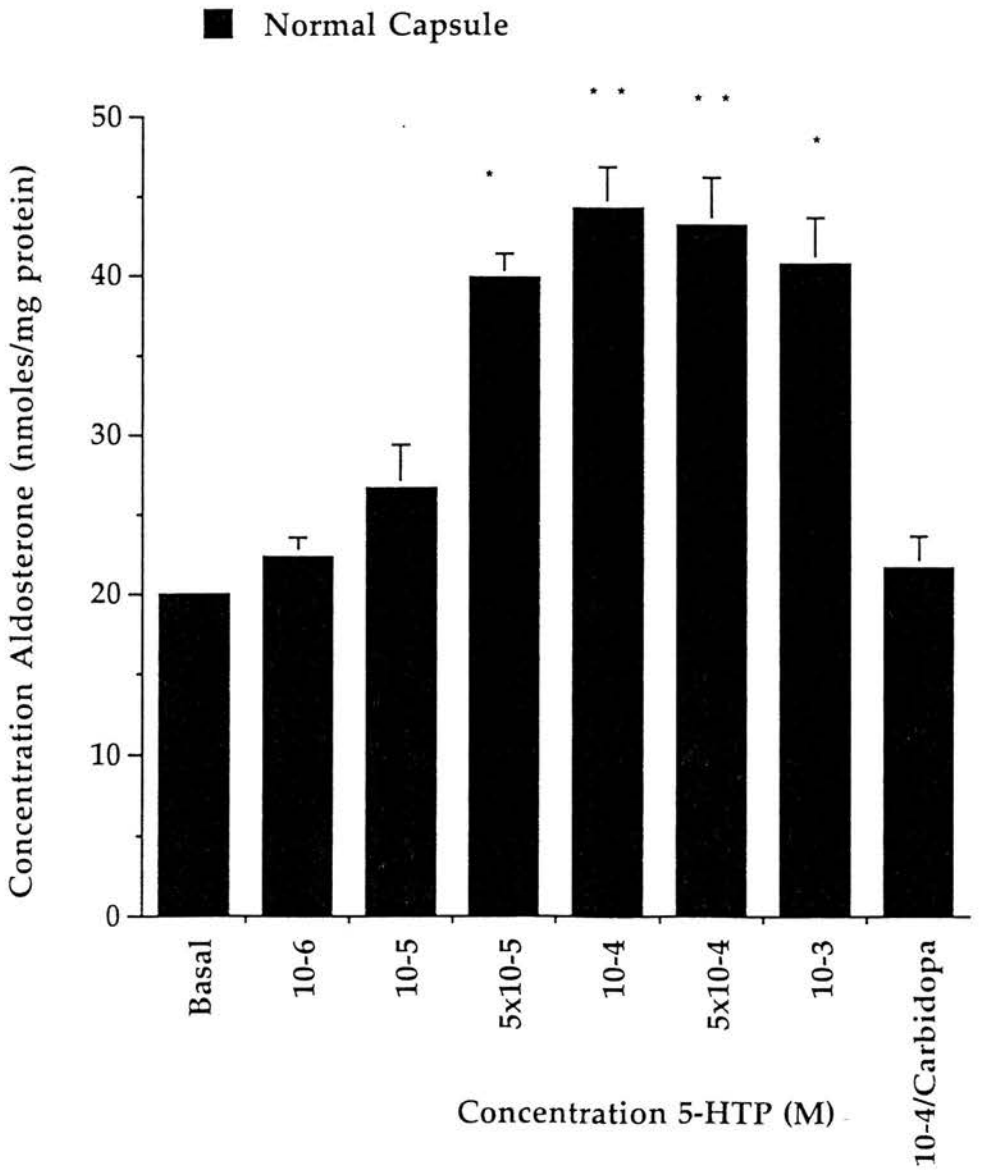


Figure 5.1. *Dose-dependent increase in aldosterone secretion from whole adrenal capsule in response to increasing concentrations of 5-HTP in the presence and absence of carbidopa ($10^{-4}M$).* Whole rat adrenal capsules were incubated with increasing concentrations of 5-HTP in the presence and absence of carbidopa 10^{-4}M for 1 hour at 37°C. Aldosterone secretion into the medium was measured by RIA, and corrected for protein content. Data from 4 separate experiments, with triplicate incubations performed in each, were combined and expressed as mean±SEM. *$p<0.05$ and **$p<0.01$ compared to basal levels.

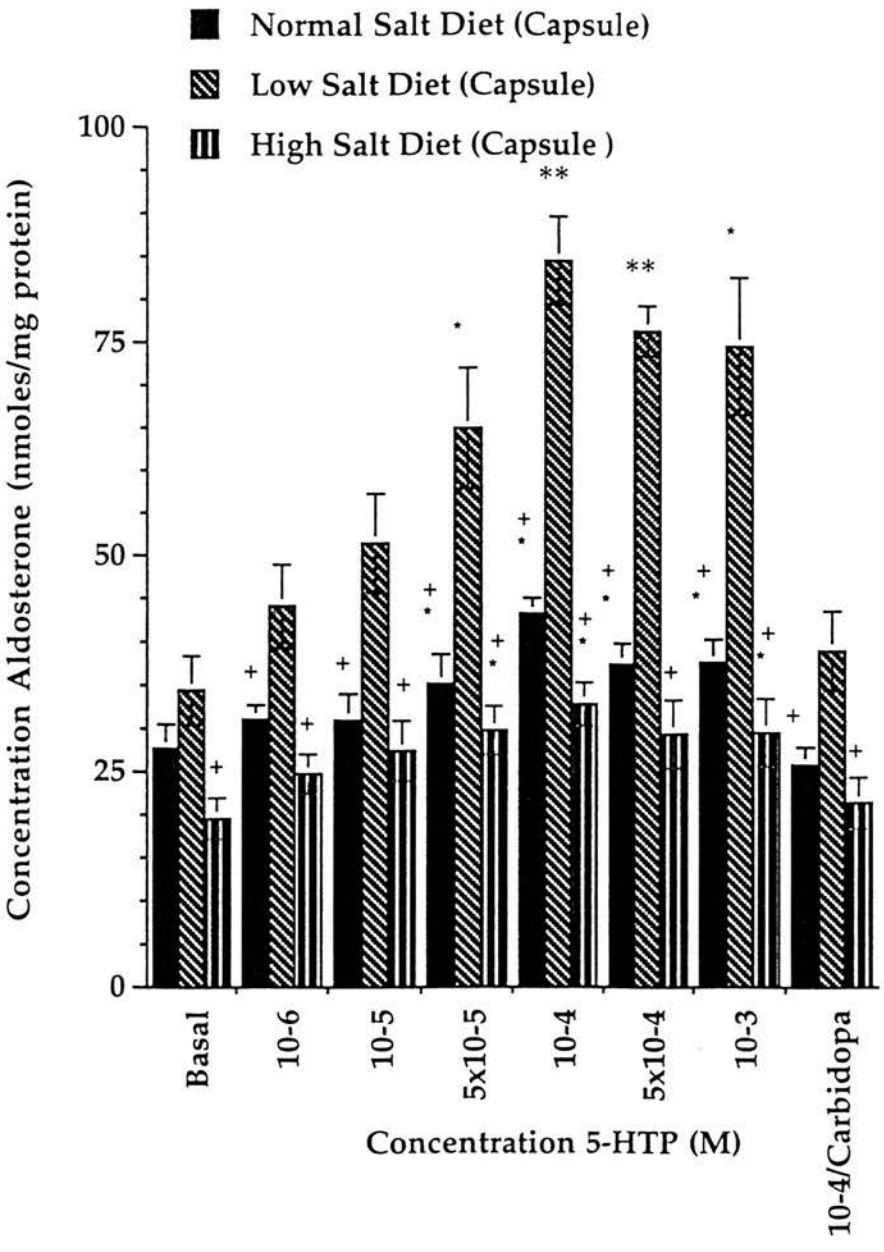


Figure 5.2. *Dose-dependent increase in aldosterone secretion from whole adrenal capsule, prepared from rats fed on a normal, low or high salt diet, in response to increasing concentrations of 5-HTP in the presence and absence of carbidopa (10^{-4}M).* Female Wistar rats were maintained on a normal (1%), low (0%) or high (3%) salt diet for one week prior to removal of their adrenal glands. Whole rat adrenal capsules were incubated with increasing concentrations of 5-HTP in the presence and absence of carbidopa 10^{-4}M for 1 hour at 37°C. Aldosterone secretion into the medium was measured by RIA, and corrected for protein content. Data from 4 separate experiments, with triplicate incubations performed in each, were combined and expressed as mean±SEM. *$p<0.05$ and **$p<0.01$ compared to basal levels. +$p<0.05$ compared with low salt diet of the same concentration.

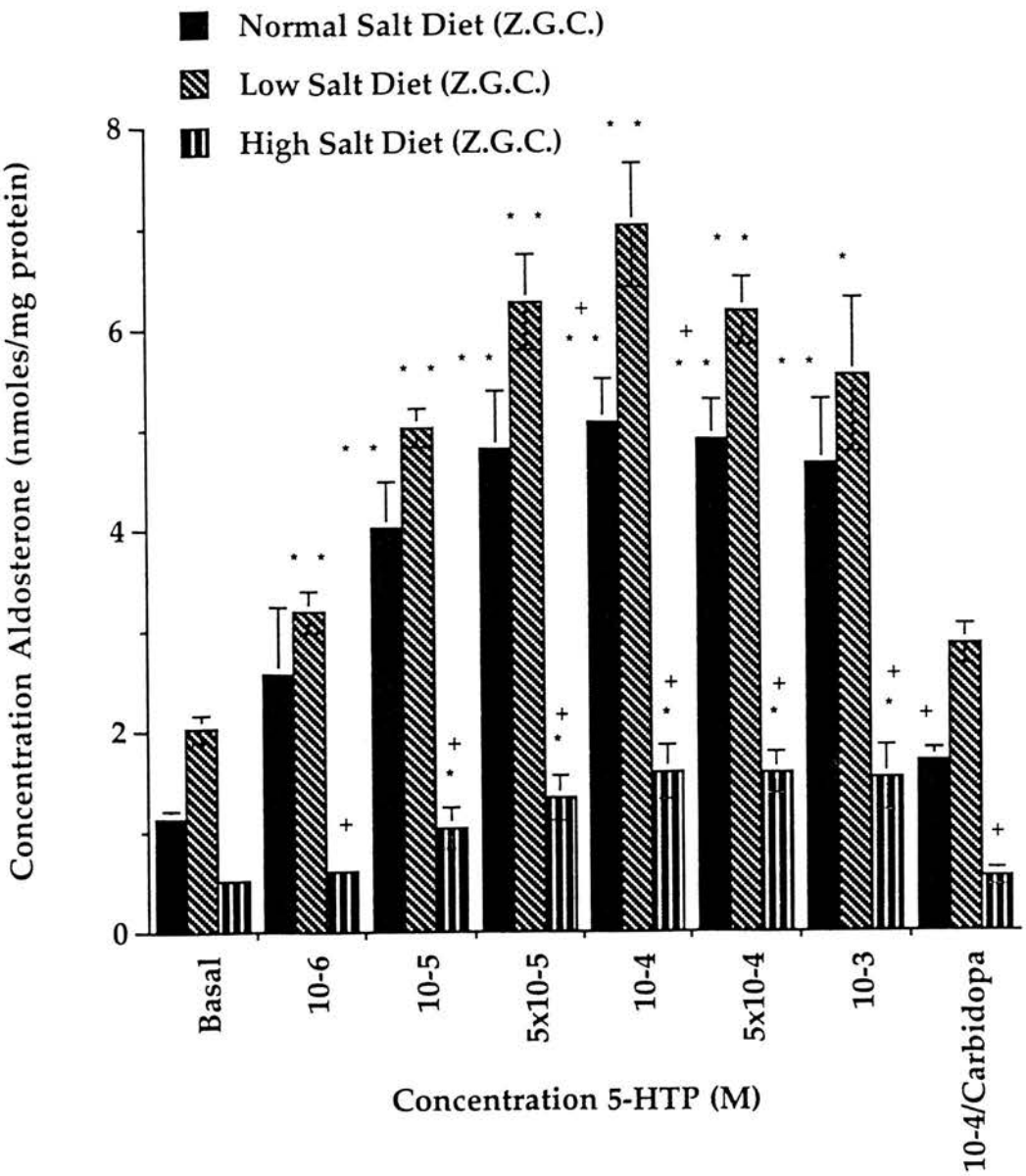


Figure 5.3. *Dose-dependent increase in aldosterone secretion from isolated ZG cells, prepared from rats fed on a normal, low or high salt diet, in response to increasing concentrations of 5-HTP in the presence and absence of carbidopa (10^{-4}M).* Female Wistar rats were maintained on a normal (1%), low (0%) or high (3%) salt diet for one week prior to removal of their adrenal glands. Isolated ZG cells were incubated with increasing concentrations of 5-HTP in the presence and absence of carbidopa 10^{-4}M for 1 hour at 37°C. Aldosterone secretion into the medium was measured by RIA, and corrected for protein content. Data from 4 separate experiments, with triplicate incubations performed in each, were combined and expressed as mean±SEM. *$p<0.05$ and **$p<0.01$ compared to basal levels. +$p<0.05$ compared with low salt diet of the same concentration.

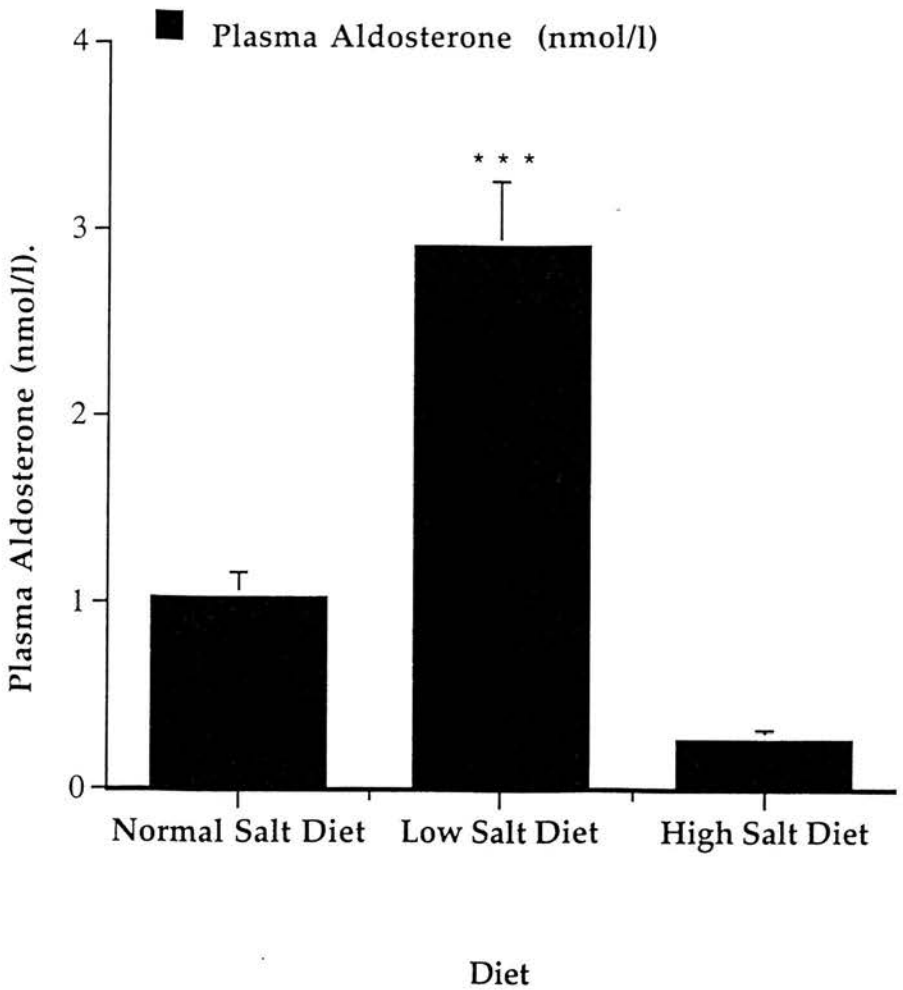


Figure 5.4. (a) *Plasma aldosterone levels measured in rats maintained on a normal, low or high salt diet for one week.* Values are mean±SEM, n=4. ***p<0.001 as compared with normal salt diet levels.

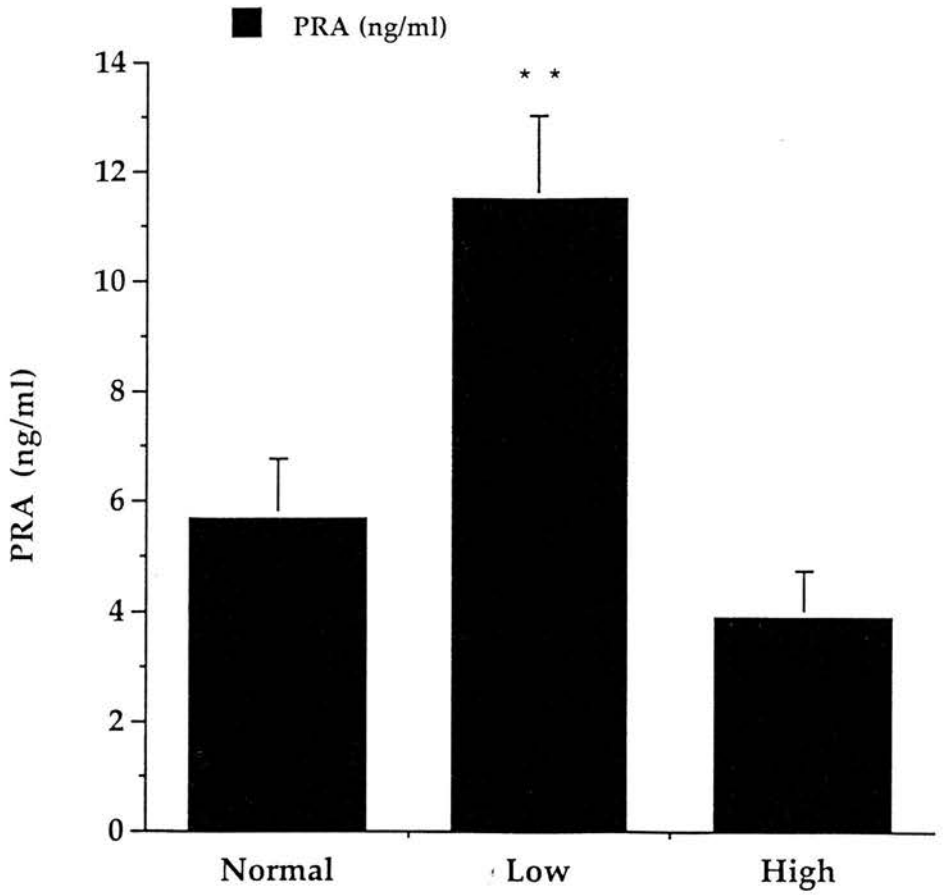


Figure 5.4. (b) *Plasma renin levels measured in rats maintained on a normal, low or high salt diet for one week.* Values are mean±SEM, n=4. **p<0.01 as compared with normal salt diet levels.

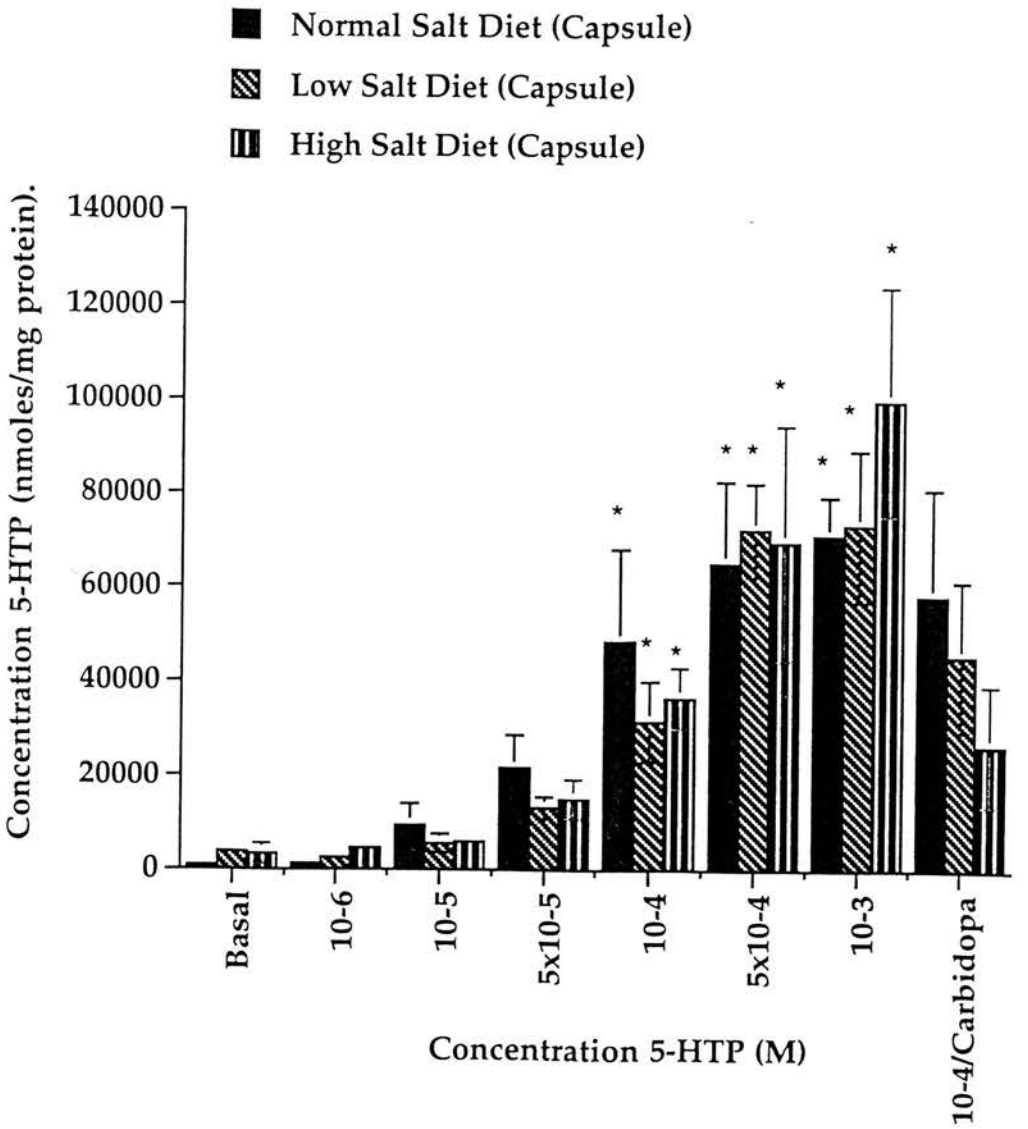


Figure 5.5. (a) *Concentration of 5-HTP measured by HPLC present in the medium after incubating whole adrenal capsules, prepared from rats fed on a normal, low or high salt diet, with increasing concentrations of 5-HTP in the presence and absence of carbidopa (10^{-4}M).* Female Wistar rats were maintained on a normal (1%), low (0%) or high (3%) salt diet for one week prior to removal of their adrenal glands. Whole adrenal capsules were incubated with increasing concentrations of 5-HTP in the presence and absence of carbidopa 10^{-4}M for 1 hour at 37°C. The concentration of 5-HTP in the medium after the incubation period was measured by HPLC, and corrected for protein content. Data from 4 separate experiments, with triplicate incubations performed in each, were combined and expressed as mean±SEM. *$p<0.05$ compared to basal levels.

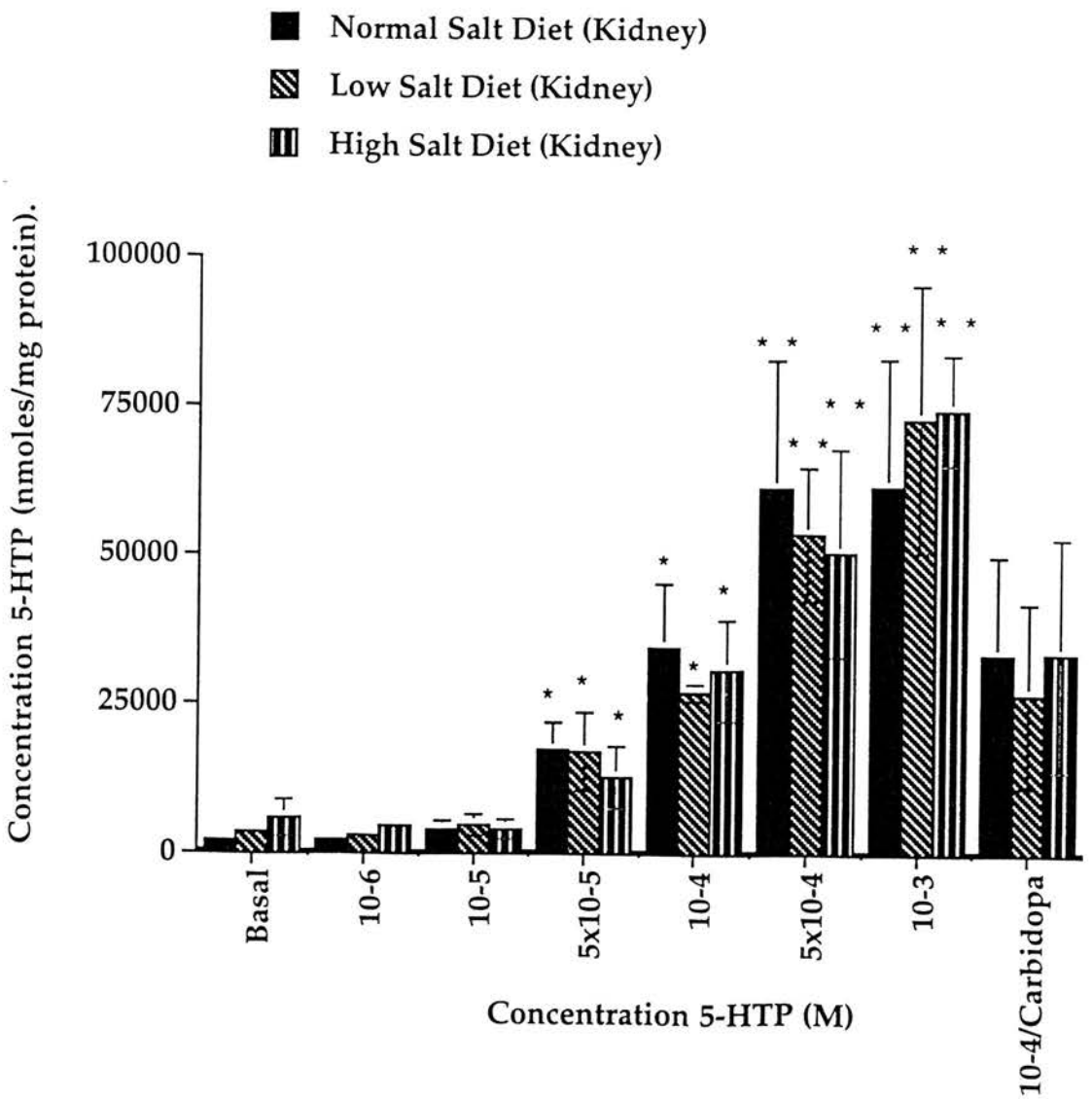


Figure 5.5. (b) *Concentration of 5-HTP measured by HPLC present in the medium after incubating sections of kidney cortex, prepared from rats fed on a normal, low or high salt diet, with increasing concentrations of 5-HTP in the presence and absence of carbidopa ($10^{-4}M$).* Female Wistar rats were maintained on a normal (1%), low (0%) or high (3%) salt diet for one week prior to removal of their kidneys. Sections of kidney cortex were incubated with increasing concentrations of 5-HTP in the presence and absence of carbidopa 10^{-4}M for 1 hour at 37°C. The concentration of 5-HTP in the medium after the incubation period was measured by HPLC, and corrected for protein content. Data from 4 separate experiments, with triplicate incubations performed in each, were combined and expressed as mean±SEM. *$p<0.05$ and **$p<0.01$ compared to basal levels.

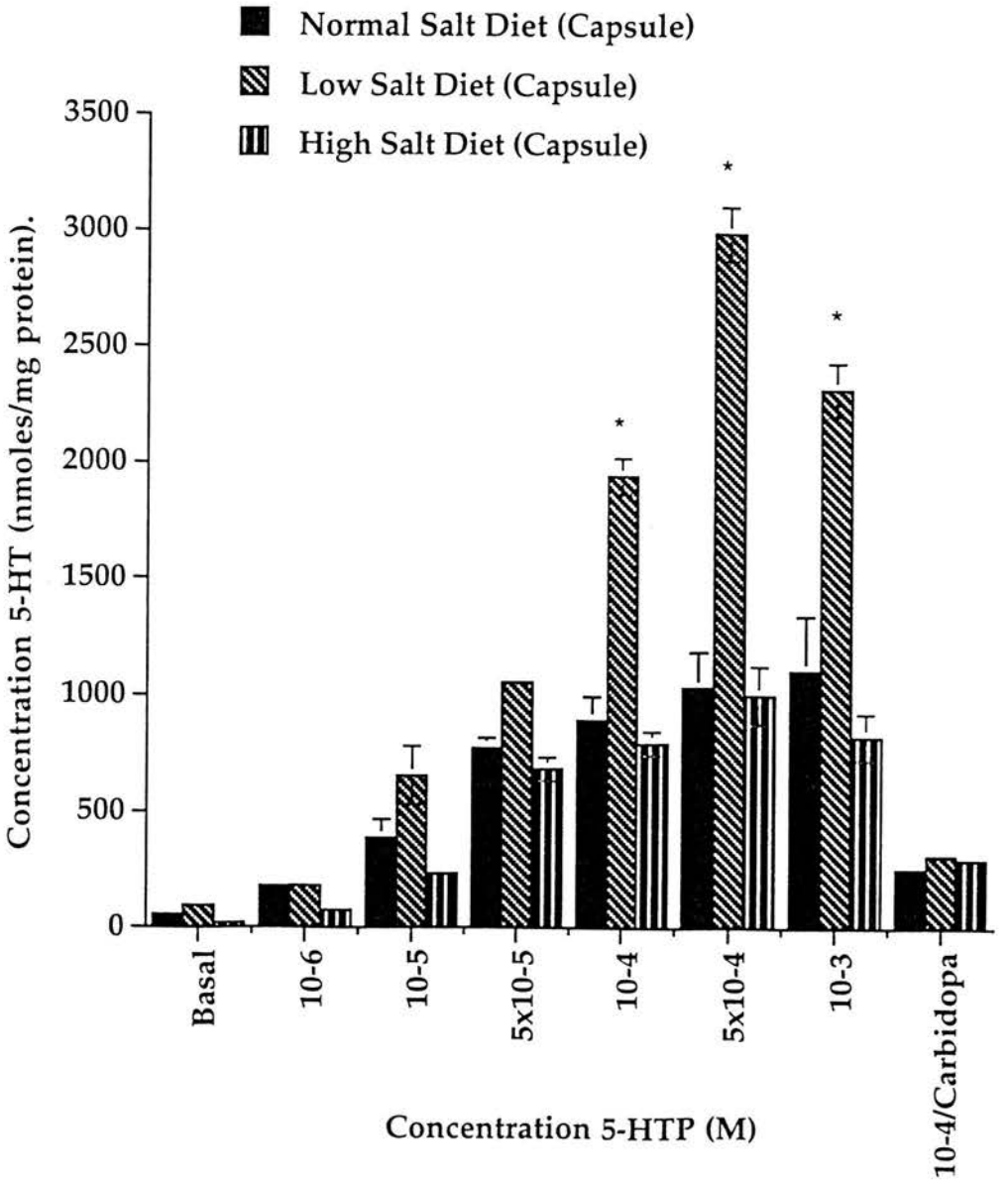


Figure 5.6. (a) *Concentration of 5-HT measured by HPLC present in the medium after incubating whole adrenal capsules, prepared from rats fed on a normal, low or high salt diet, with increasing concentrations of 5-HTP in the presence and absence of carbidopa (10^{-4}M).* Female Wistar rats were maintained on a normal (1%), low (0%) or high (3%) salt diet for one week prior to removal of their adrenal glands. Whole adrenal capsules were incubated with increasing concentrations of 5-HTP in the presence and absence of carbidopa 10^{-4}M for 1 hour at 37°C. The concentration of 5-HT in the medium after the incubation period was measured by HPLC, and corrected for protein content. Data from 4 separate experiments, with triplicate incubations performed in each, were combined and expressed as mean±SEM. *$p<0.05$ compared to basal levels.

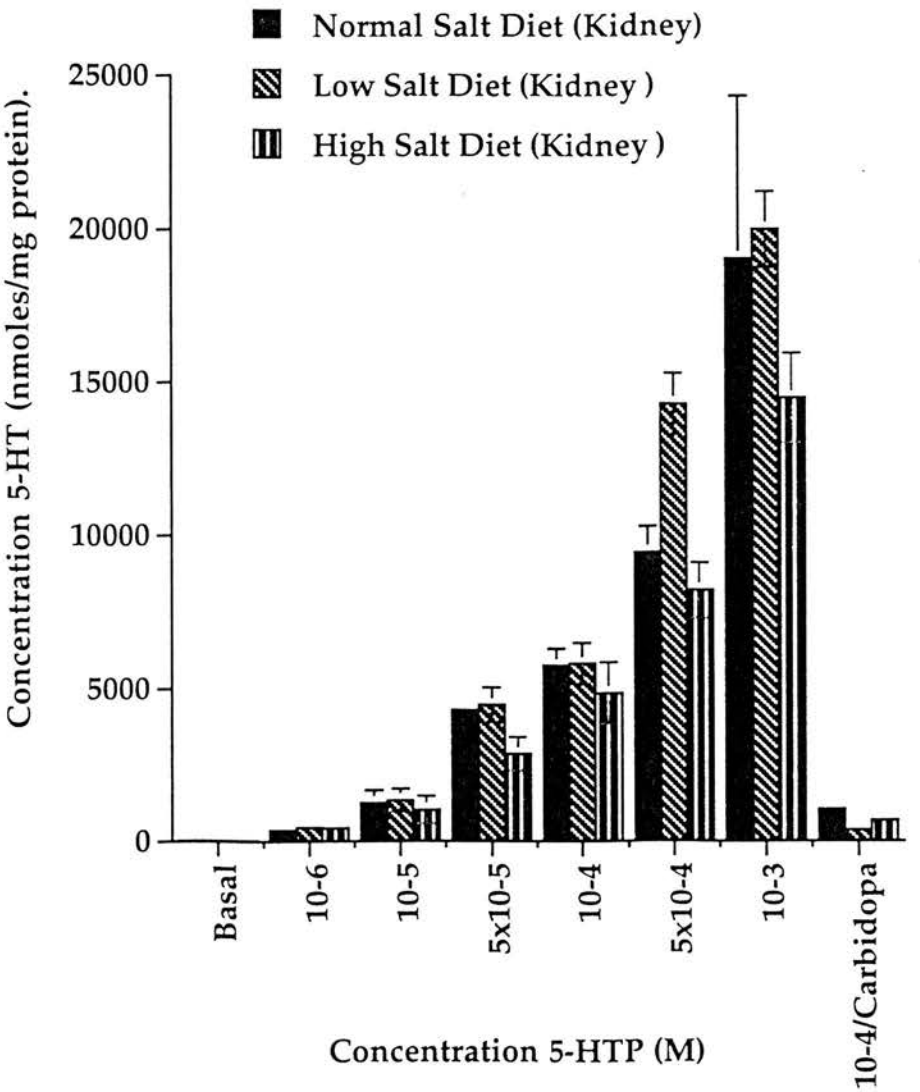


Figure 5.6. (b) *Concentration of 5-HT measured by HPLC present in the medium after incubating sections of kidney cortex, prepared from rats fed on a normal, low or high salt diet, with increasing concentrations of 5-HTP in the presence and absence of carbidopa (10^{-4}M).* Female Wistar rats were maintained on a normal (1%), low (0%) or high (3%) salt diet for one week prior to removal of their kidneys. Sections of kidney cortex were incubated with increasing concentrations of 5-HTP in the presence and absence of carbidopa 10^{-4}M for 1 hour at 37°C. The concentration of 5-HT in the medium after the incubation period was measured by HPLC, and corrected for protein content. Data from 4 separate experiments, with triplicate incubations performed in each, were combined and expressed as mean±SEM.

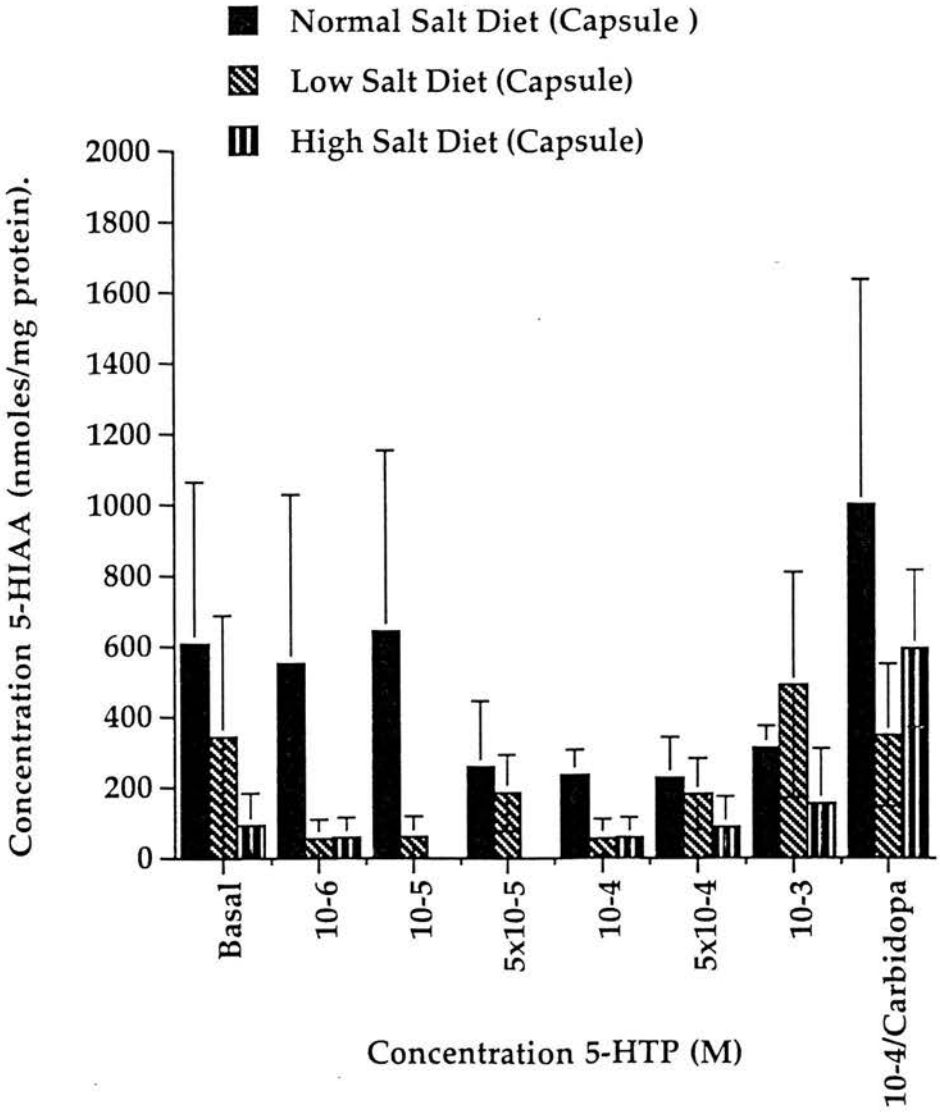


Figure 5.7. (a) *Concentration of 5-HIAA measured by HPLC present in the medium after incubating whole adrenal capsules, prepared from rats fed on a normal, low or high salt diet, with increasing concentrations of 5-HTP in the presence and absence of carbidopa (10^{-4}M).* Female Wistar rats were maintained on a normal (1%), low (0%) or high (3%) salt diet for one week prior to removal of their adrenal glands. Whole adrenal capsules were incubated with increasing concentrations of 5-HTP in the presence and absence of carbidopa 10^{-4}M for 1 hour at 37°C. The concentration of 5-HIAA in the medium after the incubation period was measured by HPLC, and corrected for protein content. Data from 4 separate experiments, with triplicate incubations performed in each, were combined and expressed as mean±SEM.

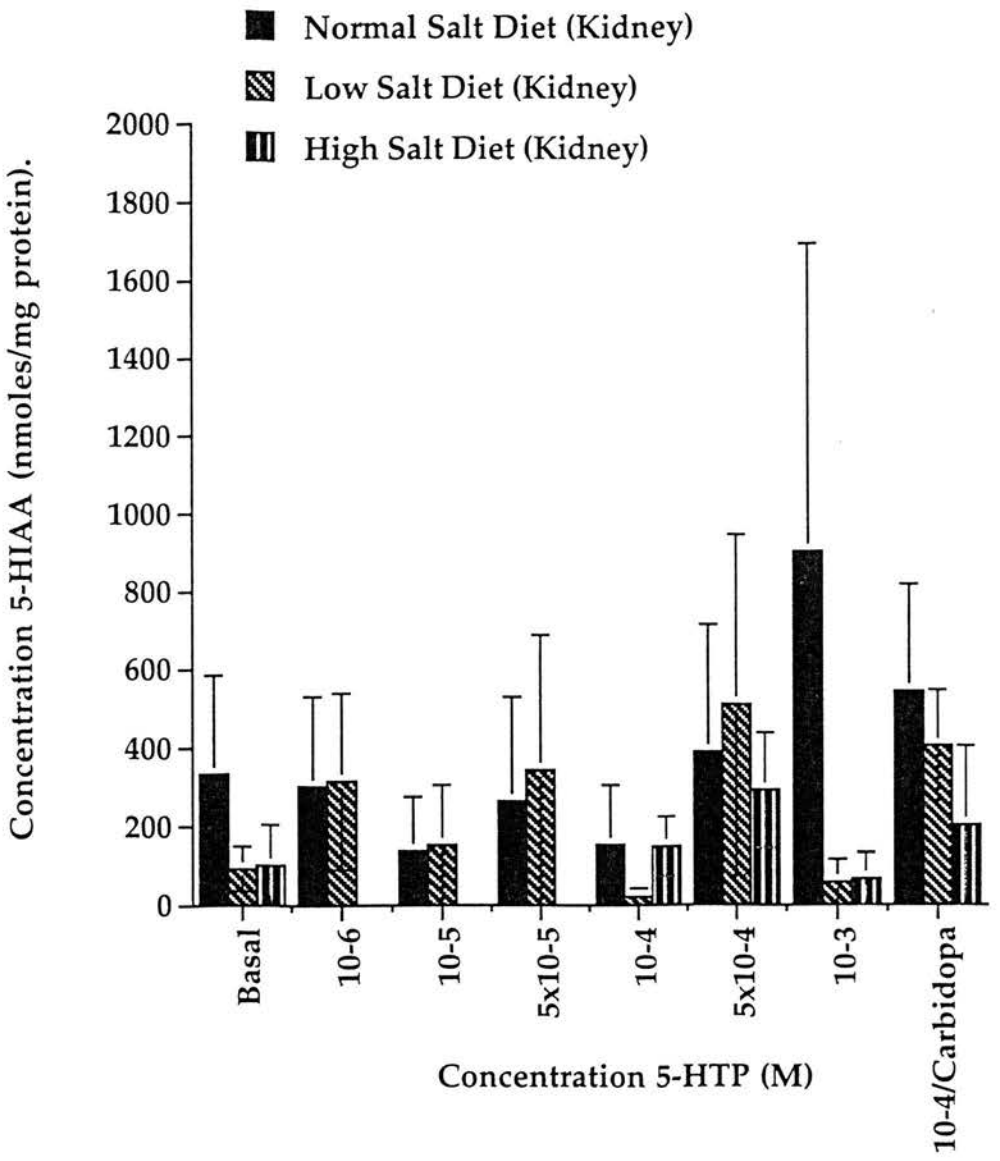


Figure 5.7. (b) *Concentration of 5-HIAA measured by HPLC present in the medium after incubating sections of kidney cortex, prepared from rats fed on a normal, low or high salt diet, with increasing concentrations of 5-HTP in the presence and absence of carbidopa (10^{-4}M).* Female Wistar rats were maintained on a normal (1%), low (0%) or high (3%) salt diet for one week prior to removal of their kidneys. Sections of kidney cortex were incubated with increasing concentrations of 5-HTP in the presence and absence of carbidopa 10^{-4}M for 1 hour at 37°C. The concentration of 5-HIAA in the medium after the incubation period was measured by HPLC, and corrected for protein content. Data from 4 separate experiments, with triplicate incubations performed in each, were combined and expressed as mean±SEM.

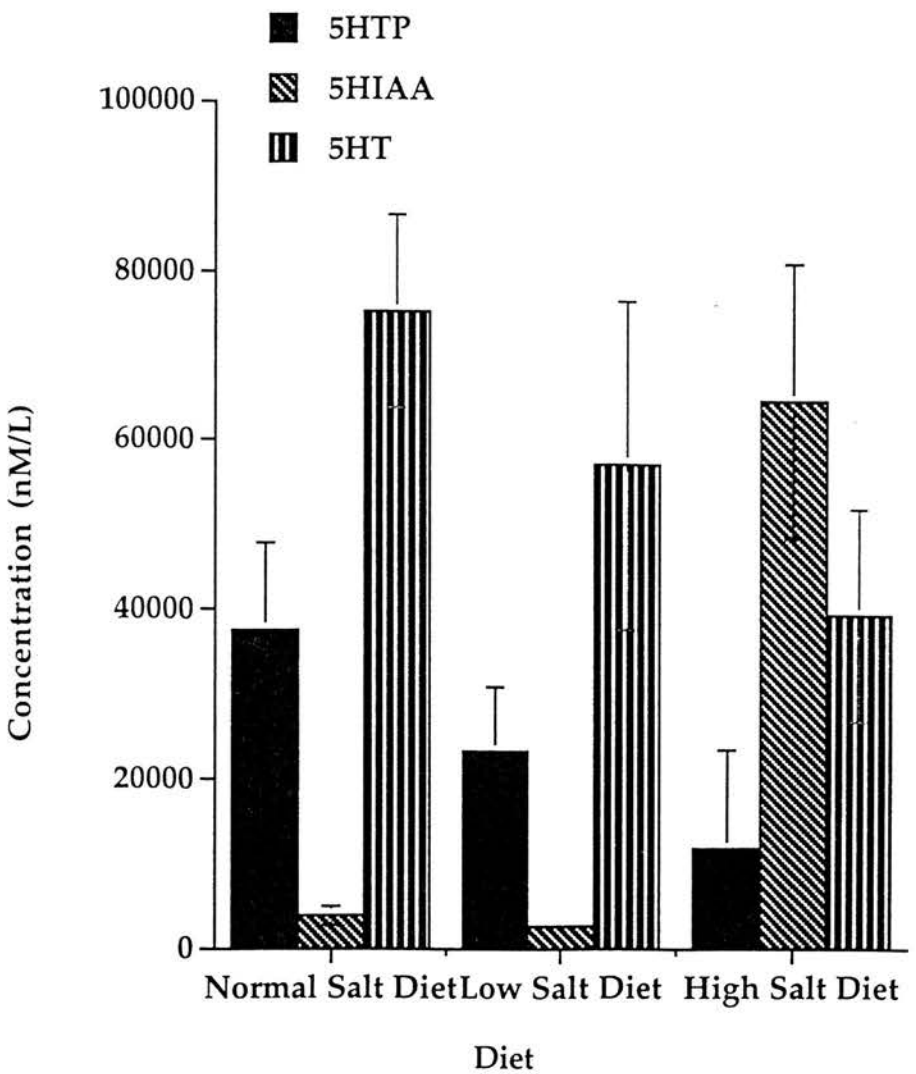


Figure 5.8. *Plasma 5-HT, 5-HTP and 5-HIAA levels measured in rats maintained on a normal, low or high salt diet for one week.* Values are mean±SEM, n=4.

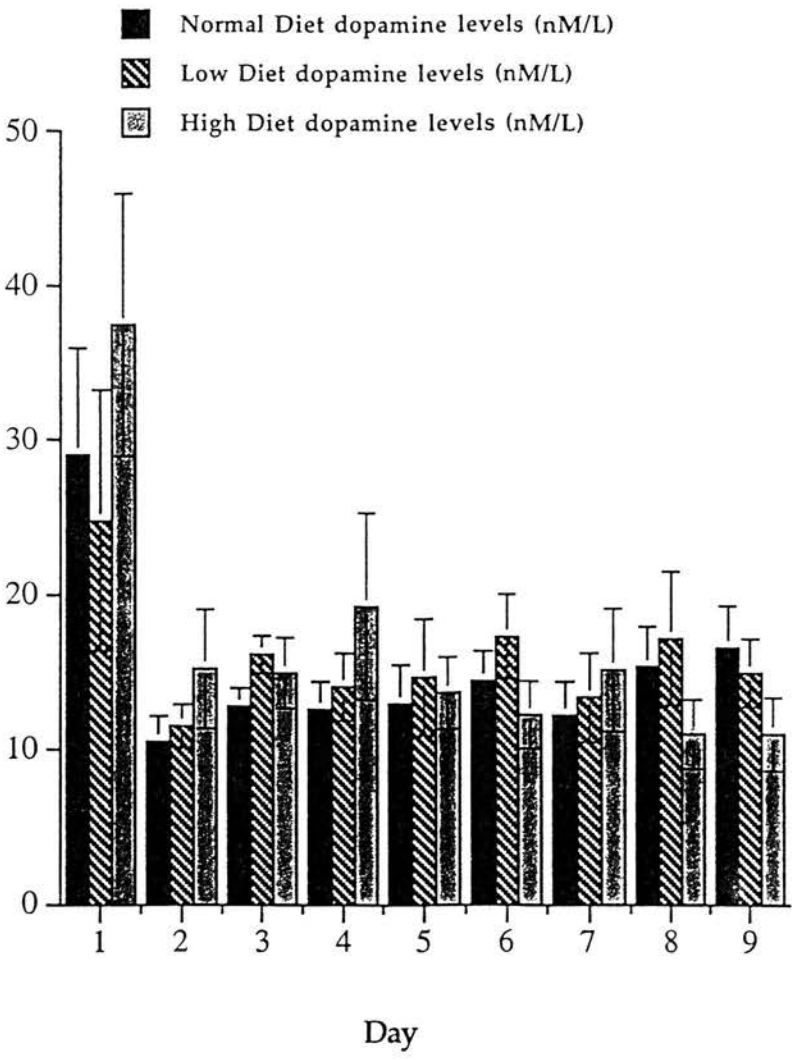


Figure 5.9. *Urinary dopamine levels measured in rats maintained on a normal, low or high salt diet for one week.* Values are mean±SEM, n=4.

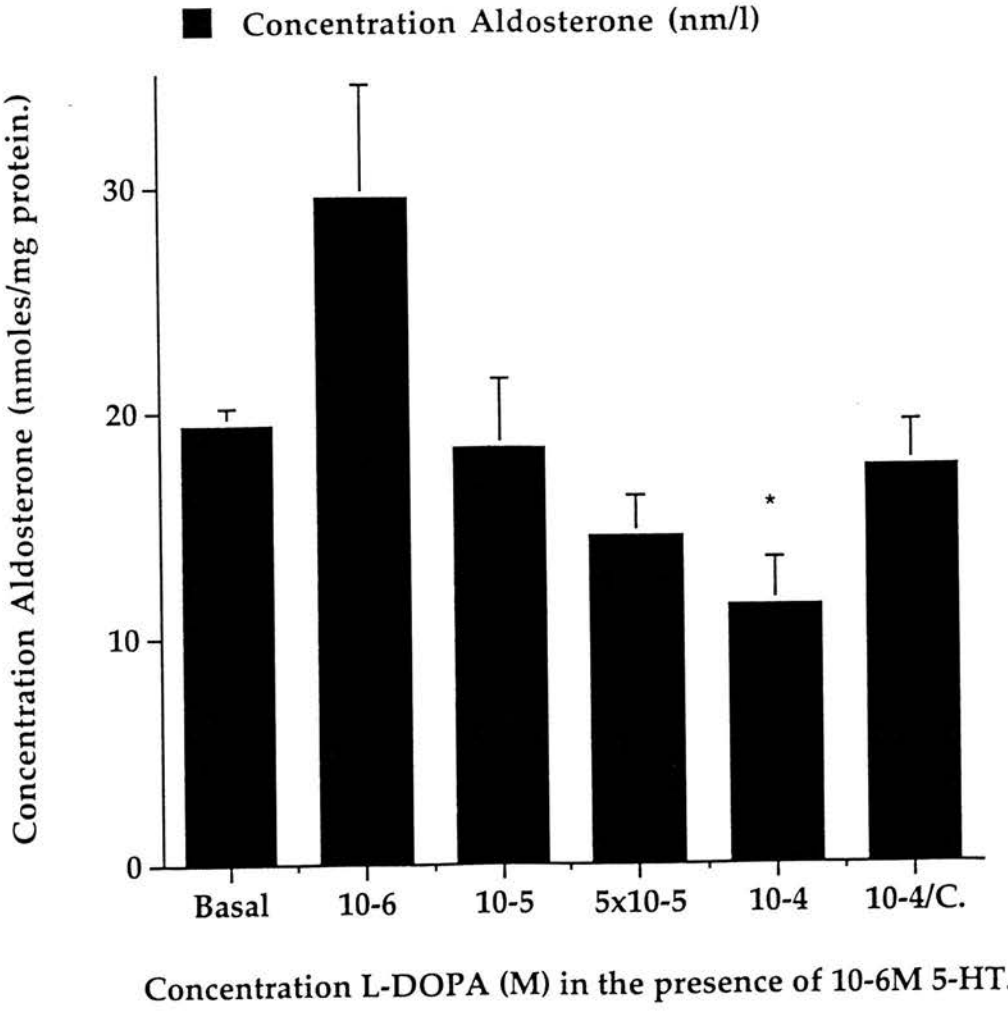


Figure 5.10. *Effects of increasing concentrations of L-DOPA on prestimulated (5-HT 10^{-6}M) whole adrenal capsules on aldosterone secretion, incubated in the presence and absence of carbidopa (10^{-4}M).* Whole adrenal capsules, prestimulated with 5-HT 10^{-6}M, were incubated with increasing concentrations of L-DOPA in the presence and absence of carbidopa 10^{-4}M for 1 hour at 37°C. After the incubation period aldosterone was measured by RIA, and corrected for protein content. Data from 4 separate experiments, with triplicate incubations performed in each, were combined and expressed as mean±SEM. Statistically significant inhibition of 5-HT-induced aldosterone secretion by L-DOPA is indicated by *$p<0.05$.

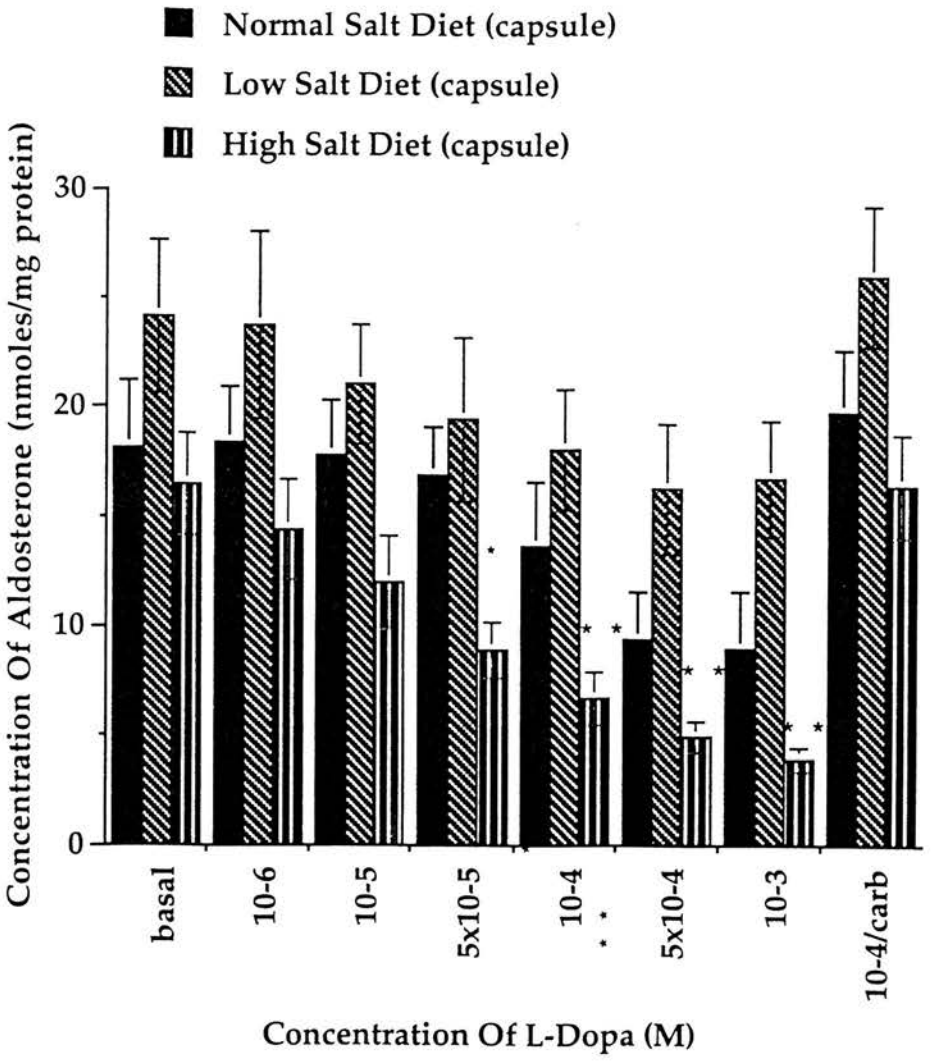


Figure 5.11. *Effects of increasing concentrations of L-DOPA on prestimulated (5-HT 10^{-6}M) whole adrenal capsules, prepared from animals maintained on a normal, low or high salt diet, on aldosterone secretion, incubated in the presence and absence of carbidopa (10^{-4}M).* Female Wistar rats were maintained on a normal (1%), low (0%) or high (3%) salt diet for one week prior to the removal of the adrenal gland. Whole adrenal capsules, prestimulated with 5-HT 10^{-6}M, were incubated with increasing concentrations of L-DOPA in the presence and absence of carbidopa 10^{-4}M for 1 hour at 37°C. After the incubation period aldosterone was measured by RIA, and corrected for protein content. Data from 4 separate experiments, with triplicate incubations performed in each, were combined and expressed as mean±SEM. Statistically significant inhibition of 5-HT-induced aldosterone secretion by L-DOPA is indicated by *$p<0.05$ and **$p<0.01$.

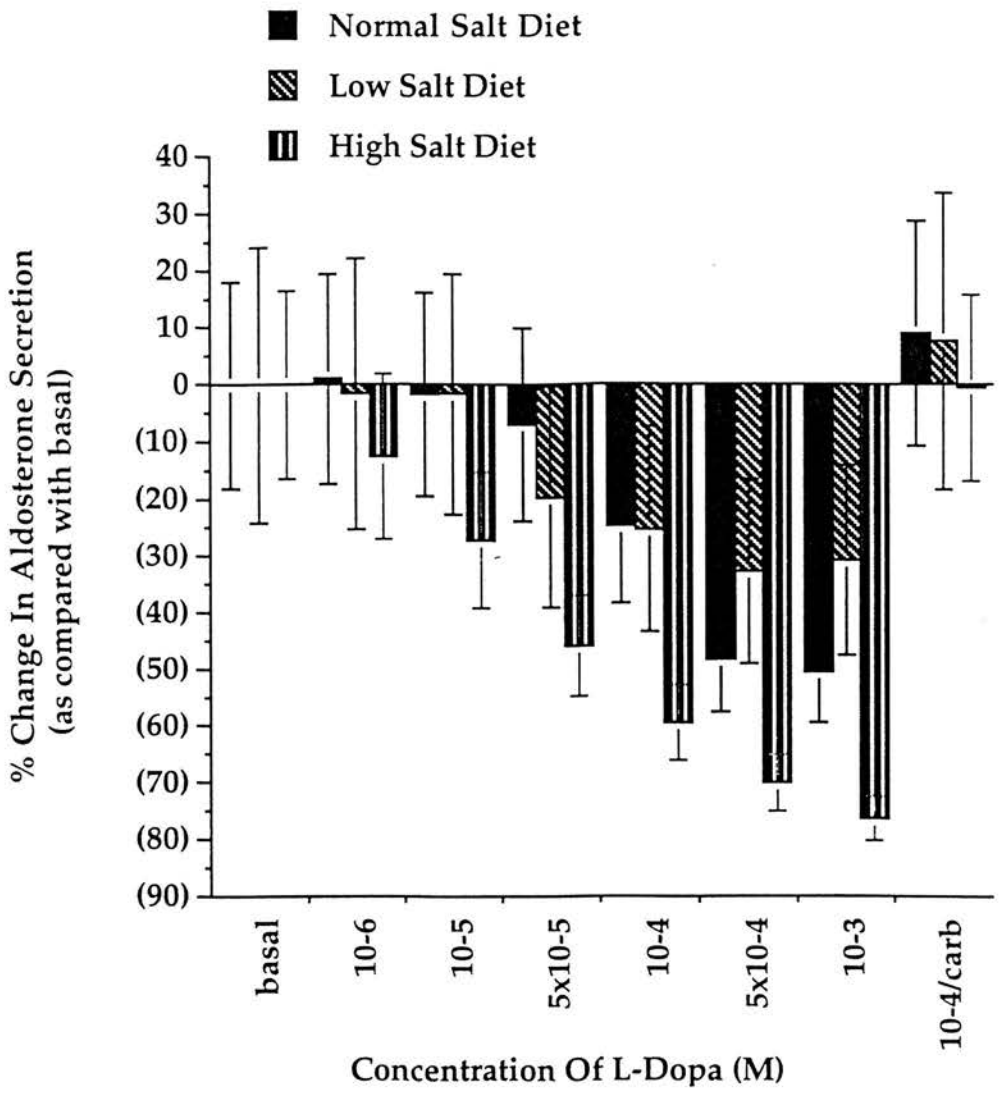


Figure 5.12. *Depicts the effects of L-DOPA as seen in Figure 5.11 as a percentage change from basal levels.* Values are mean±SEM, n=4.

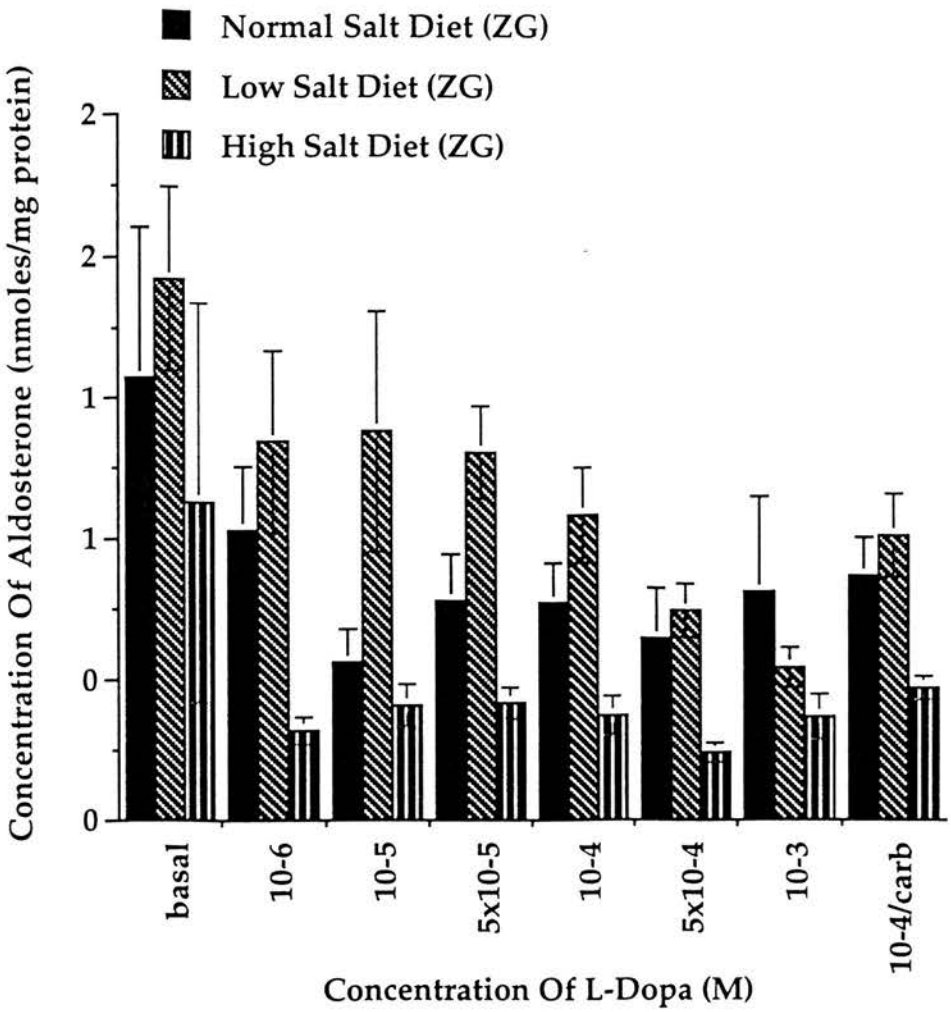


Figure 5.13. *Effects of increasing concentrations of L-DOPA on prestimulated (5-HT 10^{-6}M) isolated ZG cells, prepared from animals maintained on a normal, low or high salt diet, on corticosterone secretion, incubated in the presence and absence of carbidopa (10^{-4}M).* Female Wistar rats were maintained on a normal (1%), low (0%) or high (3%) salt diet for one week prior to the removal of the adrenal gland. Isolated ZG cells, prestimulated with 5-HT 10^{-6}M, were incubated with increasing concentrations of L-DOPA in the presence and absence of carbidopa 10^{-4}M for 1 hour at 37°C. After the incubation period aldosterone was measured by RIA, and corrected for protein content. Data from 4 separate experiments, with triplicate incubations performed in each, were combined and expressed as mean±SEM.

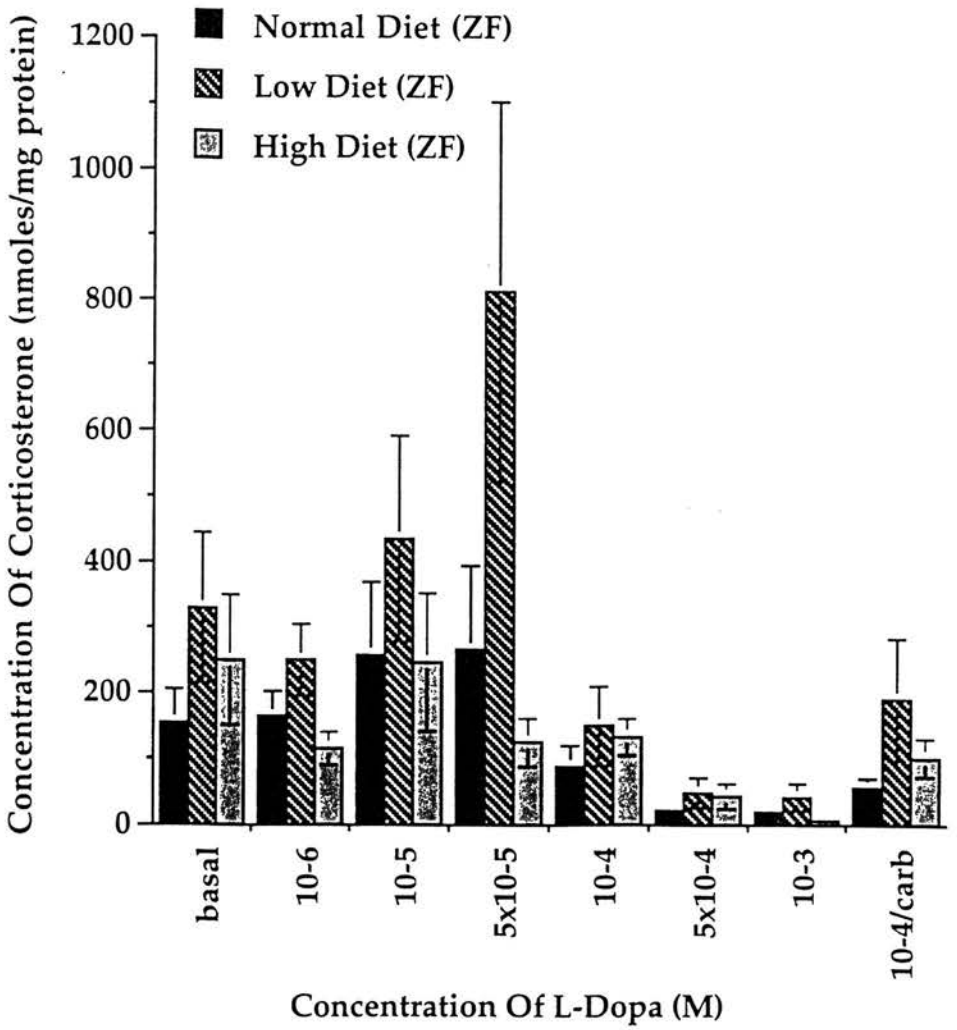


Figure 5.14. *Effects of increasing concentrations of L-DOPA on prestimulated (5-HT 10^{-6}M) isolated ZF cells, prepared from animals maintained on a normal, low or high salt diet, on corticosterone secretion, incubated in the presence and absence of carbidopa (10^{-4}M).* Female Wistar rats were maintained on a normal (1%), low (0%) or high (3%) salt diet for one week prior to the removal of the adrenal gland. Isolated ZF cells, prestimulated with 5-HT 10^{-6}M, were incubated with increasing concentrations of L-DOPA in the presence and absence of carbidopa 10^{-4}M for 1 hour at 37°C. After the incubation period aldosterone was measured by RIA, and corrected for protein content. Data from 4 separate experiments, with triplicate incubations performed in each, were combined and expressed as mean±SEM.

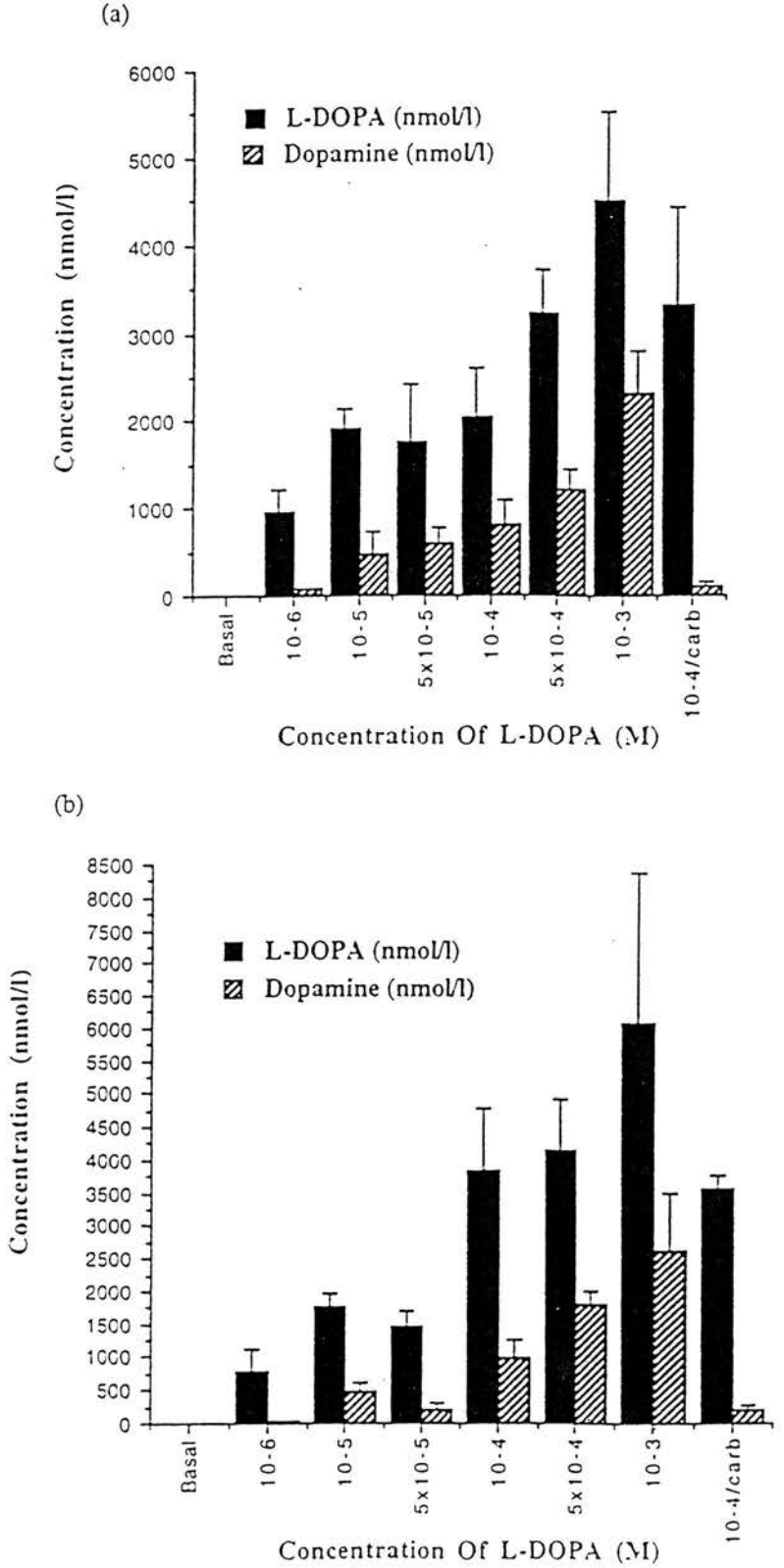


Figure 5.15. *Concentrations of dopamine measured by HPLC present in the medium after incubating prestimulated (5-HT10^{-6}M) a)adrenal capsules and b)medullary portions, prepared from animals maintained on a normal, low or high salt diet, with increasing concentrations of L-DOPA, in the presence and absence of carbidopa (10^{-4}M).* Female Wistar rats were maintained on a normal (1%), low (0%) or high (3%) salt diet for one week prior to the removal of the adrenal gland. Whole adrenal capsules or medullary portions, prestimulated with 5-HT 10^{-6}M, were incubated with increasing concentrations of L-DOPA in the presence and absence of carbidopa 10^{-4}M for 1 hour at 37°C. After the incubation period dopamine levels were measured by HPLC. Data from 4 separate experiments, with triplicate incubations performed in each, were combined and expressed as mean±SEM.

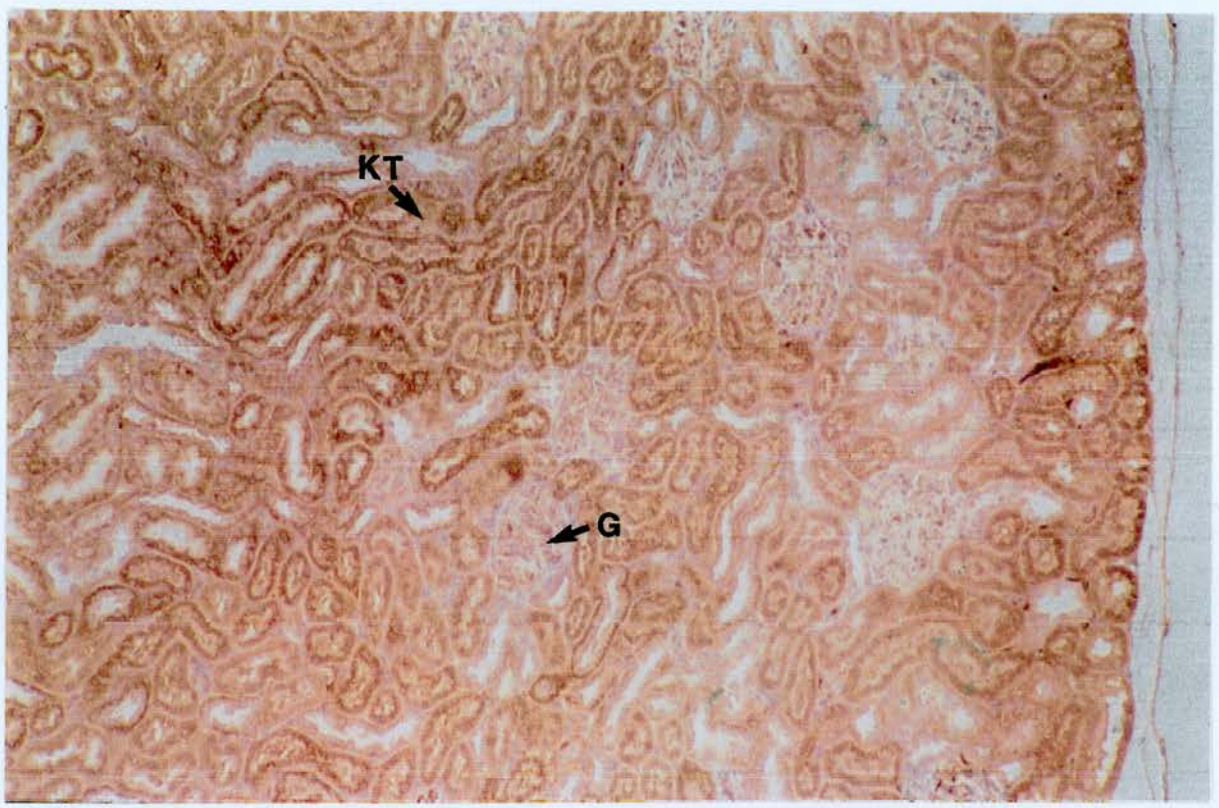


Figure 5.16 : A section through the rat kidney cortex stained with a monoclonal antibody to L-AAAD. KT = kidney tubules, G = glomeruli. Magnification = x200.

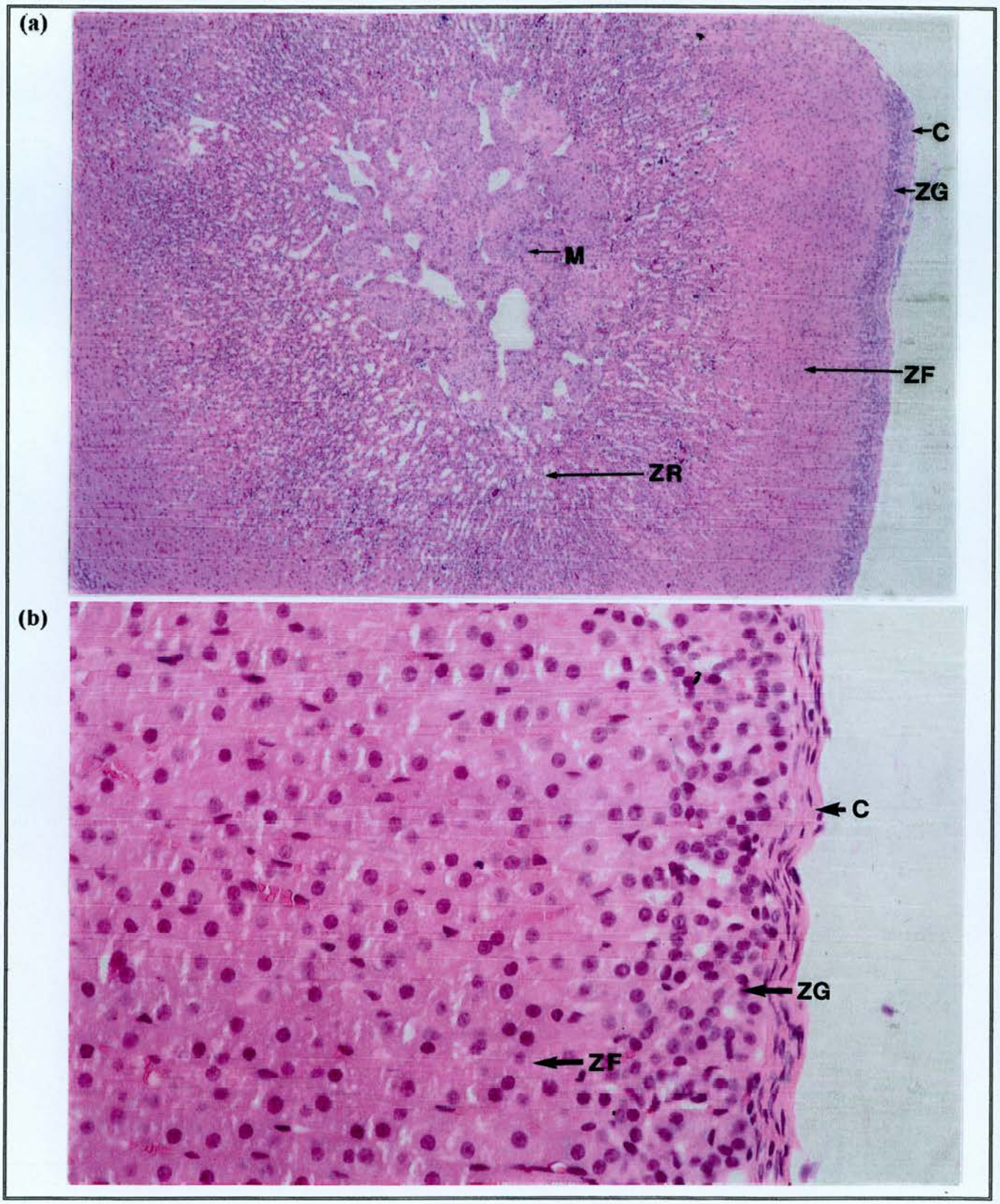


Figure 5.17 : A section through the rat adrenal gland showing general morphology stained with H/E. (a) magnification x80, (b) magnification x200. C=capsule, ZF=zona fasciculata, ZG=zona glomerulosa and M=medulla.

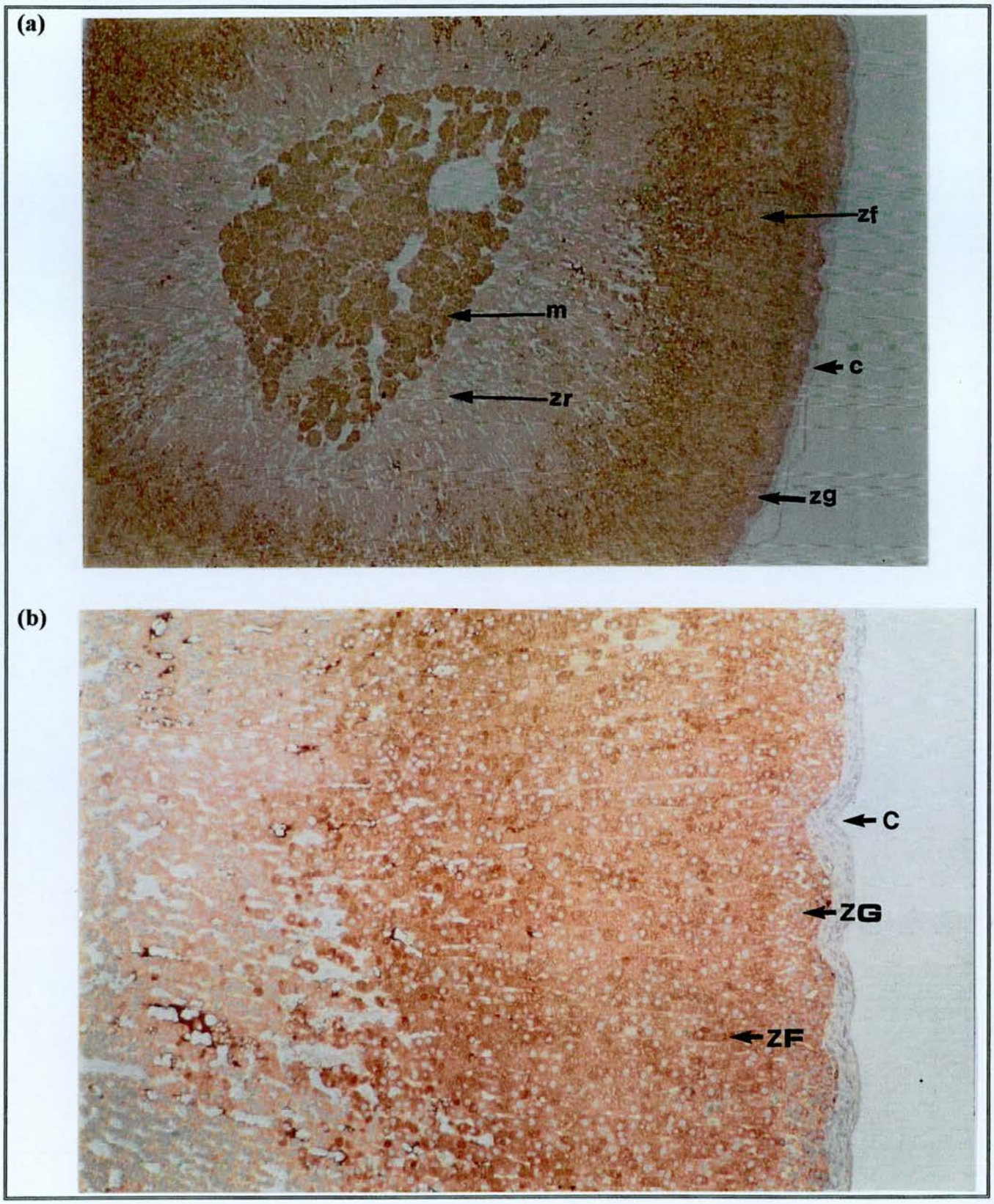


Figure 5.18 : A section through the rat adrenal gland showing L-AAAD immunoreactivity. (a) magnification x80, (b) magnification x200. C=capsule, ZF=zona fasciculata, ZG=zona glomerulosa and M=medulla.

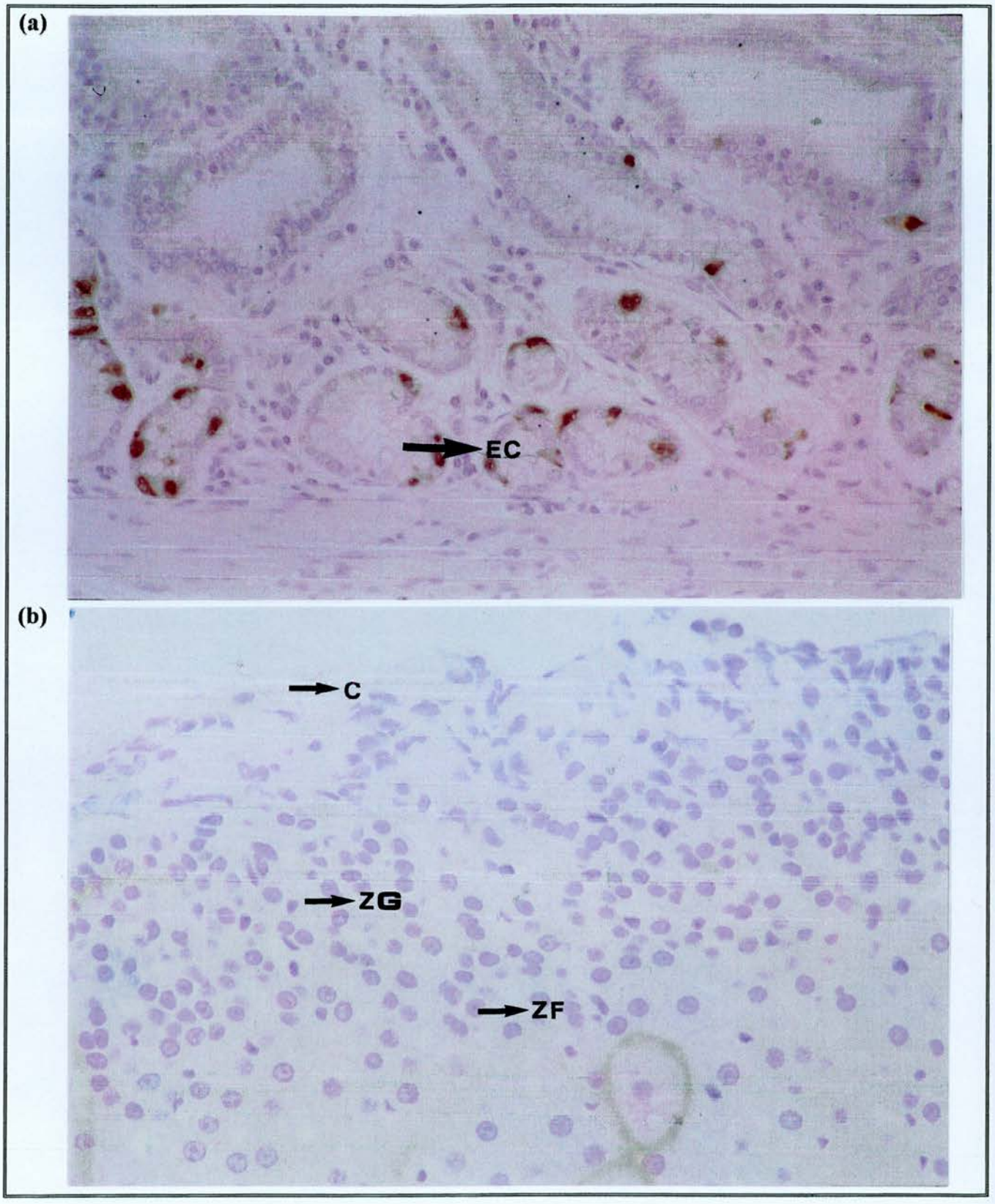
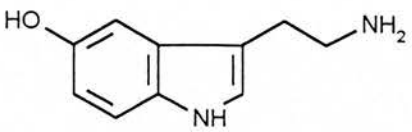


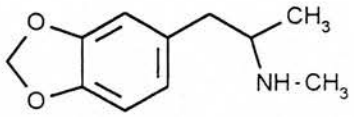
Figure 5.19 : 5-HT immunoreactivity present within, (a) the rat gut, (b) the rat adrenal cortex. C=capsule, ZF=zona fasciculata, ZG=zona glomerulosa and EC=enterochromaffin cells. Magnification = x500.

SUBSTRATES AND INHIBITORS FOR THE 5-HT TRANSPORTER

SUBSTRATES

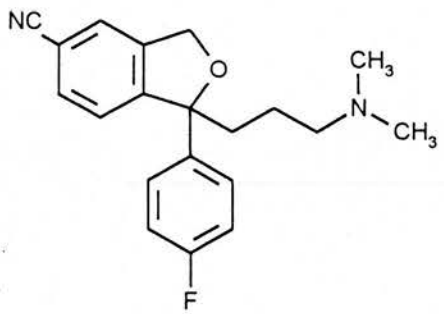


5-HT

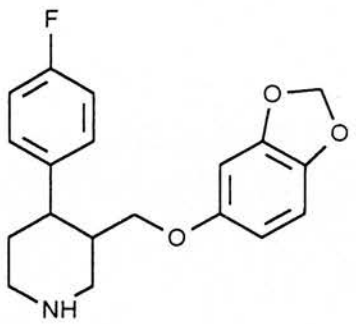


Methylenedioxymethamphetamine
(MDMA, 'Ecstasy')

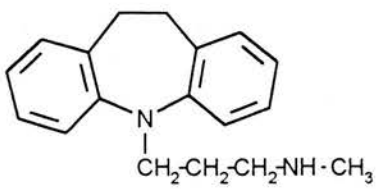
INHIBITORS



Citalopram



Paroxetine



Desmethylimipramine (DMI)

5.4. Discussion

Aldosterone is the main mineralocorticoid found in man, responsible for the regulation of electrolyte balance by stimulating active sodium transport and promoting secretion of potassium ions in the kidney and several other epithelial tissues, for example, sweat glands, intestinal mucosa and salivary glands (Morris, 1981). The production of aldosterone by the cells of the ZG in the adrenal cortex, is under multifactorial control, regulatory factors include plasma potassium levels, AII, ACTH and ANH (Porter *et al.*, 1992). Under normal physiological conditions the most important regulator of aldosterone is the RAS system, which produces the effector peptide AII. In states of sodium depletion, the sensitivity of the adrenal ZG response to the stimuli AII, potassium ions and ACTH is increased. In the case of AII, there is an increase on receptor density and circulating levels of AII are also elevated due to the effect of the low sodium status which activates renin release from the juxtaglomerular cells of the renal cortex. Studies carried out in isolated ZG cells from the rat demonstrated that the sensitivity of the aldosterone response to 5-HT was increased if the rats were maintained on a low sodium diet (Al-Dujali *et al.*, 1982). As expected, altering salt intake in the rats used in this study significantly altered plasma aldosterone concentrations (Figure 5.1). The lower the salt content of the diet, the greater the concentrations of aldosterone measured, as a result of increased mineralocorticoid output from the adrenal cortex, in order to maintain physiologically acceptable electrolyte levels.

The biogenic amines, dopamine and 5-HT, have both been shown to modulate aldosterone secretion. Although unlike the regulators mentioned above their roles are

poorly understood. The idea of both 5-HTergic and dopaminergic control over aldosterone secretion is of particular interest because dopamine and 5-HT are known to have reciprocal effects upon renal sodium handling in the kidney (Itskovitz *et al.*, 1988). Hence, reciprocal effects upon aldosterone secretion from the adrenal cortex would therefore be complementary to this.

Dopamine, 5-HT, 5-HTP and 5-HIAA output in the urine of rats from the different salt diets were measured. There was no significant change in the output of any of these compounds over the time course of the diets, or a difference between values from each of the diet groups. Blood levels of 5-HT, 5-HTP and 5-HIAA from animals on the different salt diets were also measured, and the results were not significantly different between the salt diet groups. Dopamine levels and L-DOPA levels were not able to be measured due to the failure of the HPLC system, however, as urine levels are directly proportional to plasma concentrations, it is likely that once measured these results would also not be significantly different from each other. This would seem to indicate that dopamine and 5-HT levels are not altered by changes in sodium status, and suggest that if dopamine or 5-HT do indeed function as physiological regulators of aldosterone biosynthesis they do not act via the circulation.

Many studies have confirmed that 5-HT acts to stimulate aldosterone secretion from the ZG of the adrenal cortex from a variety of species. In contrast the inhibitory effect of dopamine on aldosterone secretion is still to be fully understood. An inhibitory effect of dopamine on aldosterone secretion was first proposed by Muller in 1979. Dopamine was shown to attenuate AII stimulated aldosterone secretion by bovine

adrenal cells (McKenna *et al.*, 1979). Furthermore, investigations with metoclopramide, a dopamine antagonist, were shown to increase plasma aldosterone levels in normal adults (Carey *et al.*, 1982), an effect which could be attenuated or abolished through concurrent administration of dopamine (Brown *et al.*, 1982). This effect could not be blocked by infusion of dexamethasone, saralasin or converting enzyme inhibitors, suggesting a specific dopaminergic mechanism is involved (Sowers *et al.*, 1986; Stern *et al.*, 1981). Experiments using isolated ZG cells have supported the idea that this effect is through dopamine antagonism at the adrenal level (Stern *et al.*, 1986).

The negative control of aldosterone secretion is through inhibition of agonist stimulated cAMP formation (Gallo-Payet *et al.*, 1991). Evidence from autoradiographic studies has shown the presence of D_2-like dopamine receptors within the adrenal cortex (Amenta *et al.*, 1994). Analysis of ^{3}H-spiroperidol binding to sections of the human adrenal cortex has shown that the most powerful competitor to binding was clozapine, indicating the presence of a D_4 receptor within the adrenal gland, negatively coupled to adenylyl cyclase (Caccavelli *et al.*, 1992; Sokoloff & Schwarz, 1995). No binding of the D_1- ligand ^{3}H-SCH23390 was reported in the human, in contrast to earlier reports in rat adrenal glands (Missale *et al.*, 1986).

The origin of 5-HT and dopamine in relation to their actions on the cortex is still under investigation. Circulating levels of 5-HT are low, (less than 1nmole/L), as 5-HT is mainly confined to platelets were it is stored, and also any infused 5-HT is rapidly taken up by platelets where it is stored (Humphrey & Toh, 1954). Free 5-HT is constantly

being cleared from the circulation through a number of mechanisms, including uptake by endothelial cells of the lung, inactivation in the liver and uptake by platelets (Gershon & Tamir, 1985). It has been reported that the dose of 5-HT needed to overcome the rapid uptake by platelets constitutes a lethal dose in the rat (Liddle, Island & Meador, 1962). Circulating dopamine is also too low (0.3-12nmol/l) to reach the high concentrations required in *in vitro* studies (10-100mmol/l) to inhibit aldosterone secretion (Vizi *et al.*, 1993). Thus local delivery, or production of 5-HT and dopamine from precursors, within or around the adrenal cortex is necessary to allow the high concentrations required to elicit an effect.

In platelets 5-HT was originally thought to be biologically inert unless it was released when platelets aggregate at the site of tissue damage.. In a study by Osim *et al.*, (1983), platelets from three species of animals were obtained. The platelets were labelled with ^{111}In oxine to tag individual platelets and with ^{14}C-labelled 5-HT. In brief, this study showed that platelet 5-HT was not inert and that in fact it could be deposited in three major organs, one of which was the adrenal gland. The mechanism for this deposition remains unclear. Hinson *et al.*, (1989), demonstrated the presence of mast cells within the walls of arterioles which penetrate the capsular tissue of the adrenal gland in the rat. These cells are appropriately positioned for their products to reach the subcapsular arterioles which are thought to be the site at which blood flow through the gland is controlled. Since mast cells can store 5-HT, it was proposed that these mast cells could be a local source of 5-HT. Dopamine infused into the rat for 30 minutes resulted in significantly raised plasma levels of dopamine, but the levels present within the adrenal gland remained unchanged (Buu & Lussier, 1990). This study did

demonstrate that L-DOPA was readily taken up by the rat adrenal cortex, which was accompanied by a rapid rise in dopamine levels seen in the adrenal cortex. The L-DOPA increase was specific to the cortex, however dopamine levels increased both in the cortex and the medulla. Local noradrenergic varicose axon terminals in the ZG of the rat were found to be capable of taking up dopamine from the circulation, and to then release it or convert it to noradrenaline, providing the possibility of a fine tuning of the local circulation and aldosterone biosynthesis (Vizi, *et al.*, 1993).

Evidence has now accumulated from many studies for the close colocalisation of adrenocortical and medullary cells, within the adrenal gland of many species, which may form the basis of many interactions between the two endocrine systems. Uptake of 5-HT by adrenaline-storing cells has been observed in the rat and mouse adrenal medulla (Kent *et al.*, 1984; Sudar *et al.*, 1979). In the rat adrenal, rays of medullary tissue have been reported to extend across the outer zones of the cortex, up to the capsular zone, some of these rays follow the connective fibres of the large adrenal vein. Even after cell isolation the interaction between the two cell types was visualised (Gallo-Payet, *et al.*, 1987). Chromaffin cells have also been revealed in pig and human adrenal gland projecting from the medulla across all three cortical zones (Bornstein, *et al.*, 1991, 1994). The fact that 5-HT has been localised within chromaffin cells of the adrenal medulla in the frog, rat, human and mouse adrenal gland, and can be released under splanchnic nerve stimulation, gives rise to the hypotheses that this medullary 5-HT may be the local source of the amine 5-HT in the regulation of aldosterone secretion from the ZG (Delarue *et al.*, 1988).

The possibility of local production of both 5-HT and dopamine within the adrenal cortex of various species has been hypothesised by various groups. Plasma concentrations of both 5-HTP and L-DOPA are sufficiently high to support the idea that 5-HT and dopamine could act by a paracrine mechanism, via changes in L-AAAD activity, to modulate aldosterone secretion. The idea that 5-HTP could be converted to 5-HT either centrally, peripherally or locally, to act directly on the ZG, increasing aldosterone secretion was first reported in *in vitro* studies in isolated cells and in the whole isolated perfused adrenal gland (Muller & Zeigler, 1968; Haning *et al.*, 1970; Hinson & Vinson, 1989). Trost & Muller (1976) reported that capsular tissue metabolised 5-HT to 5-HIAA and another unidentified metabolite. 5-HTP itself has been shown to have a slight stimulatory effect on aldosterone (Chapter 3).

The presence of 5-HT and 5-HIAA in rat and frog adrenal extracts by HPLC has been reported (Holzwarth *et al.*, 1984). In a study by Delarue *et al.*, (1992), conversion of 5-HTP to 5-HT and also tryptophan to 5-HT was demonstrated in the frog inter-renal tissue. Holzwarth & Brownfield (1984), showed that L-tryptophan treatment increased 5-HT immunostaining in adrenal chromaffin cells of reserpine-depleted animals. In contrast Verhofstad & Jonsson reported that 5-HT could be formed only by decarboxylation of 5-HTP, and not by L-tryptophan administration or by a tryptophan-hydroxylase inhibitor, in the adrenal gland of rats. This is in agreement with the results presented in this study.

Both biogenic amines are converted from their precursors, 5-HTP and L-DOPA, respectively by L-AAAD. *In vitro* studies indicate that the adrenal cortex has the

ability to synthsise 5-HT and dopamine from their precursors (Delarue, 1992; Buu & Lussier, 1992), suggesting the presence of L-AAAD within the gland. Beltramo *et* al., (1993) investigated the distribution of L-AAAD immunoreactivity in the adrenal gland by utilizing a polyclonal antibody raised in rabbits against an L-AAAD recombinant protein made in *E. coli.* Their results showed strong L-AAAD immunoreactive cells present within the medulla of the mouse adrenal, a faint diffuse immunoreactivity was also evident within the ZF. No immunoreactivity was found within the ZG. This study has shown, via immunohistochemistry with a specific monoclonal antibody, L-AAAD immunoreactive cells present within the medulla, ZG and ZF of the rat adrenal.

In order to investigate whether 5-HT itself is present within the rat adrenal ZG, staining for 5-HT was performed using a specific monoclonal antibody. This staining if evident would provide evidence for potential 5-HTergic innervation. Figure 5.20b) shows a distinct lack of staining in the rat adrenal cortex, suggesting a lack of 5-HTergic innervation in this area.

In the rat adrenal capsule, aldosterone secretion was found to be significantly higher in low salt diet compared to normal salt diet when incubated with 5-HTP, this was the opposite for the L-DOPA side of things, in which the results were significantly different in animals fed on the high salt diet, with more inhibition seen. Parallel results were seen in the isolated cell experiments, however the concentration of aldosterone was approximatley ten-fold lower in the isolated cell preparations. This may be explained in terms of the collagenase digestion technique used, which may have destroyed a proportion of the ZG cells. Another factor that may affect the results is that the cells

are isolated thus removing cell to cell contact (e.g. gap junctions), between the ZG cells. The ZG cells of rats on the lower salt diet clearly show a higher capacity to secrete aldosterone in response to 5-HTP in a dose-dependent manner. This suggests that the 5-HTP is converted by the enzyme L-AAAD to 5-HT, and that this 5-HT acts to stimulate aldosterone secretion from the ZG. Inhibition of L-AAAD by carbidopa also resulted in inhibition of the aldosterone response to 5-HTP and L-DOPA. Carbidopa did not however affect basal aldosterone production. Measurement of 5-HT when incubated with 5-HTP in increasing concentrations, in both adrenal capsule and kidney cortex, show a dose-dependent increase of 5-HT production. In the case of the adrenal cortex, the increase of 5-HT production appeared more prominent with rats on a low salt diet. These results suggest that L-AAAD may be regulated by salt intake in the rat. In both the adrenal capsule and the kidney cortex, carbidopa appeared to inhibit the production of 5-HT from 5-HTP, but did not inhibit the basal levels of 5-HT produced. One area of concern would be contamination of the isolated cell preparation with ZF cells. The staining of the gland located L-AAAD immunostaining within this region, and so this may add to the effect purported to be due to the ZG. The method of isolation has been used for many years and systematically produces a cell suspension with a very low, insignificant ZF contamination (Haning *et al.*, 1970). Also the capsular preparations could act as a positive control to the isolated cell work, no ZF contamination will be present within these incubations. However, to finalise these experiments a full dose response to 5-HTP in incubations containing ZF cells alone could be carried out and any aldosterone secretion into the medium measured and subtracted from the isolated ZG results. This would also enable us to answer whether or not the effect seen is a physiologically important one.

Conversion of L-DOPA to dopamine was seen in capsular and medullary preparations, consistent with this idea of local production of 5-HT and dopamine. These results are supported by the immunohistochemical localisation of the enzyme within the rat adrenal medulla and cortex. Incubation of intact adrenal capsules, pre-stimulated with 5-HT, with increasing concentrations of L-DOPA, resulted in a significant dose dependent inhibition of aldosterone secretion, further indicating a local effect of dopamine, as L-DOPA itself has no effect on aldosterone biosynthesis. In similar experiments on capsules taken from rats on different salt diets, such an inhibitory effect was seen in all cases, and could be prevented by carbidopa, an inhibitor of L-AAAD. In isolated ZG cells, parallel results emerged. However, in these experiments, aldosterone secretion was much lower (>10 fold). This is possibly the result of the dispersion process, with some cells being destroyed by the collagenase digestion or sheer force. Perhaps the receptor sites are affected by the collagenase process also. In both capsules and cells L-DOPA appeared to inhibit aldosterone secretion from high salt diet groups more strongly (>70%), than from the normal salt diet group, which in turn was affected more than the low salt diet group.

L-AAAD has been reported to have an optimum pH of 7.2 for dopamine production and 8.3 for 5-HT biosynthesis (Rahman *et al.*, 1980). Changes in salt intake may affect the Na^+/H^+ antiport present on cell membranes of all mammalian cells studied, and offers a possible mechanism whereby changes in sodium intake may regulate enzyme activity.

The regulation of the L-AAAD enzyme remains a controversial area. The enzyme itself and its regulation are important in disease states such as Parkinson's and Alzheimer's. In fact ageing may affect brain L-AAAD activity, it has been shown that the ontogenetic evolution of L-AAAD activity is biphasic, increasing from birth to adulthood and then decreasing with age (Brus, 1975). In a study with a potent enzyme-activated irreversible inhibitor of L-AAAD, MFMD, given systemically to mice, L-AAAD activities were markedly inhibited in the brain, heart and kidney, producing a substantial and long-lasting decrease in the catecholamine and 5-HT content of these organs (Jung *et al.*, 1979). It has been shown that some physiological stimuli and compounds that affect dopamine receptors can also affect L-AAAD activity. In the rat retina L-AAAD activity increases in response to light, this change is mediated by D_1-receptors (Rossetti *et al.*, 1990). Various agonists and antagonists to the D_1- and D_2-receptor sites in rat brain have been shown to modulate L-AAAD activity, and this study hypothesised that L-AAAD activity within the rat brain may be modulated by these receptors (Zhu *et al.*, 1993). In fact, brain L-AAAD is thought to be modulated by dopamine receptors, being activated after blockade of the receptor and inhibited after stimulation of brain dopamine receptors (Li *et al.*, 1993). Coge *et al.*, (1990) reported that a direct phosphorylation via an AMP-dependent protein kinase may have a role in the regulation of L-AAAD, also altered gene expression is the mechansim responsible for the long-term regulation of L-AAAD. The results from the work presented in this chapter make a tentative hypotheses that sodium intake may be a modulator of L-AAAD activity. Further studies are needed to validate this idea, such as immunohistochemistry studies of adrenal glands taken from animals on differing salt diets.

Corticosterone levels from inner zone incubations remained essentially unaffected by the presence of L-DOPA and 5-HTP (Data not shown) except at very high concentrations. This suggests that dopamine and 5-HT preferentially affect the late pathway of steroid biosynthesis. Aldosterone synthase converts corticosterone to aldosterone and has been shown to be regulated by other steroidogenic factors such as potassium ions and sodium status (Rabattu *et al.*, 1994). With a relatively selective effect on aldosterone production, it seems likely that dopamine and 5-HT also act here. Further studies resulting from this study will be to look at the 5-HTP effect on the inner zones of the rat adrenal cortex in more detail. Initial experiments in this area have shown the general trend already reported, with a higher rate of secretion from animals on a low salt diet, although the results seen so far were not significant. Also measurement of corticosterone levels produced from the ZG and the ZF, after incubations with 5-HTP and L-DOPA would be interesting

The results of this study support the role of L-AAAD in a paracrine mechanism through which circulating L-DOPA and 5-HTP are converted to dopamine and 5-HT respectively within the ZG of the rat adrenal cortex. The locally produced dopamine will act to inhibit the biosynthesis of aldosterone, possibly through an action on the enzyme aldosterone synthase, on the late pathway of steroidogenesis. The 5-HT will act to stimulate aldosterone secretion from the ZG. Thus a local fine tuning of the adrenal cortex appears to exist with two opposing regulators of aldosterone, both present within the adrenal gland, both have receptor sites within the adrenal cortex and both have been shown to be produced locally within the ZG from their circulating

precursors. 5-HT produced and released locally within the ZG may have a role in the co-ordinated response of adrenochromaffin and adrenocortical cells during stress conditions.

Sodium status appears to affect the conversion of L-DOPA to dopamine and 5-HTP to 5-HT, suggesting that salt intake regulates the activity of L-AAAD. Eisenhofer *et al.*, (1989) demonstrated the release of plasma L-DOPA from noradrenergic neurons, reflecting neurotransmitter turnover. Previous studies have demonstrated dopamine inhibiting AII induced aldosterone secretion during sodium deficiency in humans (Drake *et al.*, 1984). The findings from studies like these and the fact that L-DOPA can be converted to dopamine within the adrenal gland, suggest a possible link between sympathetic nervous activity and aldosterone regulation. This notion is entirely compatible with an earlier suggestion that 'primary alterations in endogenous dopamine production may be responsible for physiological and pathophysiological modification of aldosterone responses to angiotensin' (Carey *et al.*, 1986). How sodium affects L-DOPA release from sympathetic neurons and its conversion to dopamine is an area to be investigated. Decreasing circulating AII by converting enzyme inhibition increases the uptake of 5-HT in the lung (Gershon *et al.*, 1985). Since AII is increased during sodium depletion, it is possible that AII may in fact inhibit 5-HT uptake and consequently increase circulating 5-HT, which then may act on the ZG. Further studies, preferably *in vivo*, are needed to fully elucidate the role of L-AAAD within the ZG, and the effect of salt intake.

The results of this study suggest that the enzyme L-AAAD, present within the rat adrenal cortex, may form part of a paracrine or autocrine mechansim for the modulation of aldosterone secretion by 5-HT and dopamine. Immunohistochemistry and biochemical studies suggest that the 5-HT that stimulates aldosterone secretion, and the dopamine that inhibits aldosterone secretion, from the rat ZG, may originate from the circulating precursors of these biogenic amines. In particular the concentrations of 5-HTP measured in rat blood were similar to those levels found to produce aldosterone secretion both in rat adrenal capsules and rat glomerulosa cells.

The presence of L-AAAD within the ZF of the rat adrenal gland requires further attention. This may be important in corticosterone modulation. The presence of the 5-HT transporter within the adrenal cortex has been determined, however as yet no evidence has been shown for the existence of a transporter for dopamine or L-DOPA. Competition experiments using an inhibitor of this system, 3-o-M-DOPA, may be able to determine the presence of such a system (Soares-da-Silva *et al.*, 1994). The possibility of changing intracellular pH as a mechanism of regulation of L-AAAD could also be investigated. The use of pH-sensitive fluorescent probes, such as BCECF, on cells taken from rats on differing salt diets would indicate if pH was affected by salt diet. The effect of manipulating the intracellular pH of isolated ZG cells on aldosterone biosynthesis, through the use of such agents such as NH_4Cl and Na^+ proprionate, could then be investigated. It may be that intracellular pH determines the relative activity of L-AAAD in producing 5-HT and dopamine. L-AAAD activity may be affected by other regulators of aldosterone secretion such as ACTH and AII. AII causes alkalinization of ZG cells, thus decreasing the intracellular pH. Could this affect the

relative synthesis of 5-HT and dopamine? Another factor is sodium status, receptor sites for both 5-HT and dopamine may be affected by sodium loading and depletion. The sodium status itself may alter intracellular pH, by activation of the RAS. L-AAAD is also important in the formation of tryptamine, which has been shown to affect aldosterone secretion from isolated ZG cells (Chapter 3). This may then have an additive effect on the response seen with 5-HTP. Another area to be investigated is the effects of noradrenaline within the ZG. Dopamine β hydroxylase is present within the ZG, which converts dopamine into noradrenaline.

Chapter Six

Role of the 5-HT Transporter in the Rat Adrenal Gland.

6.1 Introduction.

The 1960's gave the first evidence of nerve terminals taking up and sequestering monoamines, specifically norepinephrine and 5-HT, and it was soon recognised that uptake back into the nerve terminals, via transporter molecules present on the neuronal membrane, was the predominant means of inactivating monoamines that have been released into the synaptic cleft. High affinity 5-HT transporters are present on the presynaptic membranes of 5-Htergic neurons where they act to regulate the concentration of 5-HT on receptor sites. To carry out their role efficiently transporters should be present at or near sites of 5-HT release. Whether or not the 5-HT transporter molecule is uniformly distributed along plasma membranes, or if it is present only in specific areas linked with 5-HT production and release is still to be elucidated.

Inhibition of the transporter process would increase the concentration of monoamines in the synaptic cleft, thus increasing their contact with synaptic receptors. Thus antagonists of neurotransmitter transporters rank among our most important tools in biological psychiatry, for example antidepressants. On the downside they can also be potent addictive substances such as cocaine, Methylenedioxymethamphetamine (MDMA; 'Ecstasy') and amphetamines.

Depression is a wide spread clinical problem. For years the tricyclic antidepressants were the drugs of choice to treat depression, although their side effects were numerous including dry mouth, nausea, cardiac toxicity and addiction (Milne & Goa, 1992). As a deficit in 5-HTergic neurotransmission is implicated in the aetiology of clinical depression, potent and selective 5-HT reuptake inhibitors have been developed as potential antidepressant drugs, such as fluoxetine (Prozac), paroxetine (Seroxat) and

citalopram (Cipramil), marketed as having less side effects than previous remedies and non addictive. Since the advent of these new wonder drugs, a certain amount of overprescribing has occurred and as with valium and amitryptyline before these drugs have now been found to be addictive, causing in some cases more problems than the depression itself.

The 5-HT transporter itself has been implicated in the mechanism of action of various drugs of abuse, including amphetamine based drugs and MDMA. In fact prolonged ingestion of MDMA leads to the appearance of the "5-HT Syndrome" amongst other effects, caused by increased 5-HT function, leading to alterations in cognition, behaviour, autonomic nervous system function and neuromuscular activity.

Trost & Muller in 1976, in a series of experiments with radiolabelled 5-HT, first described the uptake of 5-HT into zona glomerulosa cells. Osim & Wylie in 1983 demonstrated that stored platelet 5-HT could in fact be released into other tissues. In 1994 Blakeley *et al.*, demonstrated the presence of mRNA for the 5-HT transporter within the rat adrenal gland, and since then with the use of immunohistochemistry, this group have visualised the transporter molecule specifically within the rat adrenal medulla. No traces of the transporter molecule were found within the adrenal cortex of the rat. In a study by Pahkla & Rago, (1997), utilising whole decapsulated rat adrenal gland, specific saturable [^{3}H]-citalopram binding sites were detected in the rat adrenals, with an affinity comparable to that seen in rat brain. In fact the number of binding sites seen was reported to be even higher than that reported for rat brain. In addition Schroeter *et al.*, (1997), have reported the 5-HT transporter present within epinephrine synthesizing chromaffin cells of the rat adrenal medulla. No transporter protein was detected in the ZG or the capsule.

5-HT has been shown to affect adrenal gland physiology, specifically as mentioned before the release of aldosterone from the ZG. To date no evidence has been reported as to the existence of a 5-HT transporter within the rat adrenal cortex. The source of 5-HT within the gland is still to be elucidated, although the studies detailed in Chapter 5 may in part explain one source of 5-HT within the adrenal gland. Another source may be chromaffin cell 5-HT, which could be captured from the blood, as occurs in the transporter mediated uptake of 5-HT by platelets (Gillis & Pitt, 1982).

Further to these studies, immunohistochemical studies with two antibodies to the 5-HT transporter and $\{^3H\}$ paroxetine autoradiography, were utilised to visualise the transporter within the rat adrenal gland. Also superfusion and isolated cell work with 5-HT reuptake inhibitors were carried out to define a role for the transporter within the rat adrenal gland. Finally the drug of abuse MDMA, known to act on the 5-HT transporter, was studied both *in vivo* and *in vitro*, in the rat, to elucidate the effects of MDMA ingestion on the adrenal gland.

6.2. Statistical Analysis.

Statistical significance was calculated in the individual experiments by Student's t-test for unpaired samples. A p value of <0.05 was considered significant. NS indicates non significance. *, ** and *** indicates that $p<0.05$, $p<0.01$ and $p<0.001$ respectively.

6.3. Results.

The graphs represent the mean ± SEM of four individual experiments. Within each experiment each incubation was repeated three times.

Figure 6.1 represents a dose response to 5-HT, citalopram and DMI in isolated ZG cells. As can be seen a significant increase in aldosterone production was seen with 5-HT. No alteration in aldosterone secretion was observed with citalopram or DMI in increasing concentrations (10^{-10}-10^{-5}M).

Figure 6.2 represents dose response relationships, in isolated ZG cells, between aldosterone secretion and increasing concentrations of 5-HT, of 5-HT in the presence of 10^{-6}M citalopram and of 5-HT in the presence of 10^{-9}M citalopram. A slight increase in aldosterone secretion was observed in the presence of 10^{-9}M citalopram, and this increase was more evident in the presence of 10^{-6}M citalopram. The increase seen was not significant (n=4).

Figure 6.3 represents dose response relationships, in isolated ZG cells, between aldosterone secretion and increasing concentrations of 5-HT, of 5-HT in the presence of 10^{-9}M DMI and of 5-HT in the presence of 10^{-6}M DMI. A slight increase was again seen in the presence of 10^{-9}M DMI, which again was more evident in the presence of 10^{-6}M DMI. This was also not significant (n=4).

The effect of increasing the cell concentration of the isolated ZG cell preparation was also looked at. Incubations containing 20,000 cells, 50,000 cells and 80,000 cells approximatly were incubated as detailed above for Figures1, 2 and 3. There was no significant difference between the 20,000 and 50,000 cell incubations (n=2). However the 80,000 cell incubation produced a more marked increase in aldosterone secretion in the presence of citalopram and DMI. This was not significant for n=2, and more experiments will have to be carried out with larger amounts of cells to find out if this trend is significant. Data not shown.

Figure 6.4 represents the dose response relationship seen in whole capsular tissue superfused (rate 1ml/1min) with increasing concentrations of 5-HT (10^{-9}-10^{-5}M) or increasing concentrations of 5-HT in the presence of 10^{-9}M DMI. An increase in the secretion of aldosterone was seen with capsular tissue superfused with 10^{-6}M DMI, which was significant ($p<0.05$). Maximal aldosterone secretion was 7.7 ± 0.3 nmol/l in the presence of DMI as compared to 4.9 ± 0.6 nmol/l for 5-HT alone (10^{-5}M). This was repeated in isolated ZG cells, and there was a slight increase seen in secretion of aldosterone, although it was not significant (n=4). Data not shown.

Figure 6.5 represents the dose response relationship seen in whole capsular tissue superfused with increasing concentrations of 5-HT (10^{-9}-10^{-5}M) or increasing concentrations of 5-HT in the presence of 10^{-6}M DMI. A more significant increase was observed in aldosterone secretion from capsular tissue ($p<0.01$). Maximal aldosterone secretion was 9.3 ± 0.5 nmol/l in the presence of DMI as compared to 4.0 ± 0.4 nmol/l for 5-HT alone (10^{-5}M). Again this was repeated in isolated ZG cells and although a rise in aldosterone secretion was observed it was not significant.

Figure 6.6 represents the dose response relationship seen in whole capsular tissue superfused with increasing concentrations of 5-HT (10^{-9}-10^{-5}M) or increasing concentrations of 5-HT in the presence of 10^{-9}M citalopram. An increase in the secretion of aldosterone was seen with capsular tissue superfused with 10^{-6}M citalopram, which was significant ($p<0.05$). Maximal aldosterone secretion was 5.4 ± 0.7 nmol/l in the presence of citalopram as compared to 4.9 ± 0.6 nmol/l for 5-HT alone (10^{-5}M). This was repeated in isolated ZG cells, and there was a slight increase seen in secretion of aldosterone, although it was not significant (n=4). Data not shown.

Figure 6.7 represents the dose response relationship seen in whole capsular tissue superfused with increasing concentrations of 5-HT (10^{-9}-10^{-5}M) or increasing concentrations of 5-HT in the presence of 10^{-6}M citalopram. A more significant increase was observed in aldosterone secretion from capsular tissue ($p<0.01$). Maximal aldosterone secretion was 7.8 ± 0.3 nmol/l in the presence of citalopram as compared to 4.4 ± 0.4 nmol/l for 5-HT alone (10^{-5}M). Again this was repeated in isolated ZG cells and although a rise in aldosterone secretion was observed it was not significant.

Figure 6.8 represents A) the total binding and B) the non-specific binding observed in sections of rat adrenal gland via [3H]-paroxetine autoradiography. As can be seen binding was evident within the rat adrenal medulla (220 ± 20 fmol/mg of tissue), and no binding was evident within the adrenal cortex (7 ± 6 fmol/mg of tissue).

Figure 6.9 represents the immunohistochemical staining seen in whole adrenal gland sections from rat. The antibodies were used at a 1:200 dilution. S-240-KLH demonstrated background staining throughout the gland, with a slightly darker staining evident in the medulla (Figure 6.9A). S-387-KLH, demonstrated dark immunoreactivity within the adrenal medulla, and slight background staining throughout the adrenal cortex. Also evident were areas of dark staining within the cortex associated with the vasculature and may be due to trapped platelets (Figure 6.9B).

Figure 6.10 represents the dose response relationship seen in isolated ZG cells incubated with increasing concentrations of 5-HT (10^{-10}-10^{-5}M) or increasing

concentrations of 5-HT in the presence of 1μM MDMA. As can be seen there is no significant difference between the two dose response curves (n=4).

Figure 6.11 represents the dose response relationship seen in isolated ZG cells incubated with increasing concentrations of 5-HT (10^{-10}-10^{-5}M) or 5-HT in the presence of 10μM MDMA. There is now evident a significant increase in aldosterone secretion as compared to incubations with 5-HT alone ($p<0.01$).

Figure 6.12 represents the dose response relationship seen in whole capsular tissue superfused with increasing doses of 5-HT (10^{-10}-10^{-5}M) in the presence and absence of 1μM MDMA. A significant rise in aldosterone secretion into the medium was observed ($p<0.01$). Maximal aldosterone secretion was 9.9 ± 0.7 nmol/l in the presence of MDMA as compared to 6.3 ± 0.4 nmol/l for 5-HT alone (10^{-5}M).

Figure 6.13 represents the dose response relationship seen in whole capsular tissue incubated with increasing doses of 5-HT (10^{-10}-10^{-5}M) in the presence and absence of 10μM MDMA. A significant rise in aldosterone secretion into the medium was observed ($p<0.001$). Maximal aldosterone secretion was 10.2 ± 0.2 nmol/l in the presence of MDMA as compared to 6.3 ± 0.4 nmol/l for 5-HT alone (10^{-5}M). Incubations with MDMA alone in both capsular tissue and isolated ZG cells produced an increased aldosterone secretion. However this response was not consistent (data not shown). Also superfusion studies with isolated ZG cells again produced slight increase of aldosterone secretion in response to incubations of 5-HT in the presence of differing concentrations of MDMA, but again the increase was not significant (n=4).

Graphical Illustration

- Chapter Six -

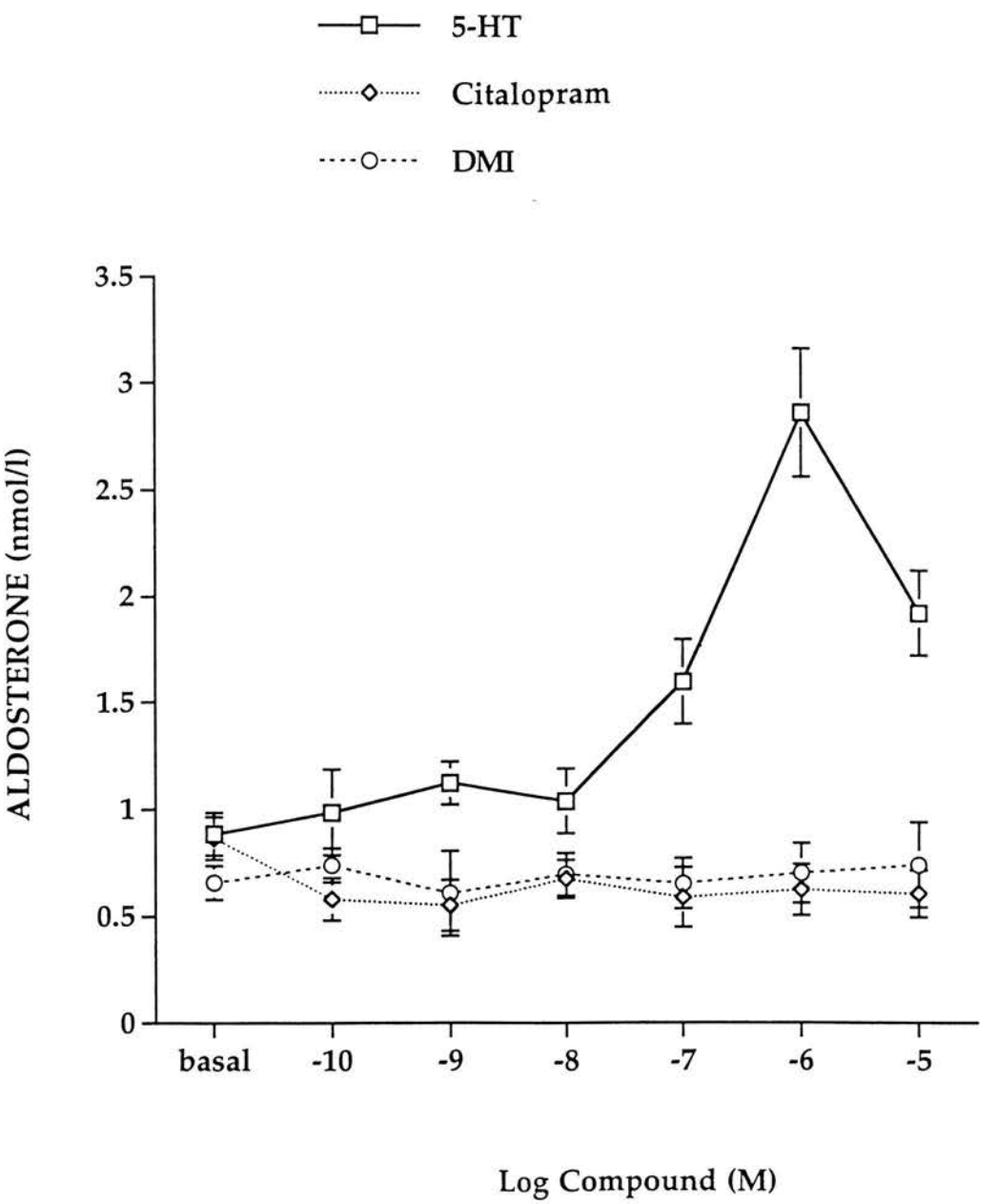


Figure 6.1. *Dose-dependent increase in aldosterone secretion from isolated ZG cells in response to increasing concentrations of 5-HT, citalopram and DMI.* Isolated rat ZG cells were incubated for 1 hour at 37°C with increasing concentrations of 5-HT, citalopram and DMI. Aldosterone secretion into the medium was measured by RIA. Data from 4 separate experiments, with triplicate incubations performed in each, were combined and expressed as mean±SEM.

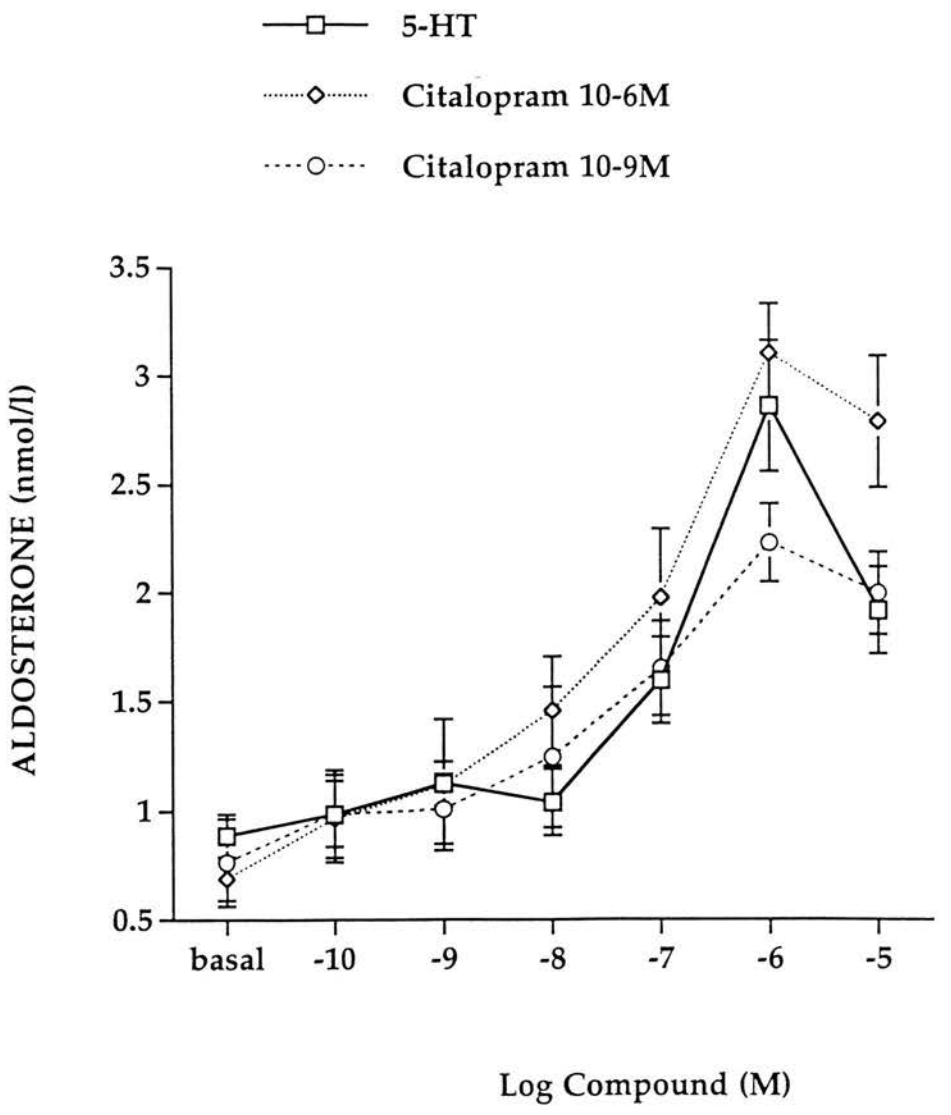


Figure 6.2. *Dose-dependent increase in aldosterone secretion from isolated ZG cells in response to increasing concentrations of 5-HT, in the presence and absence of citalopram 10^{-6}M and citalopram 10^{-9}M.* Isolated rat ZG cells were incubated for 1 hour at 37°C with increasing concentrations of 5-HT alone or 5-HT in the presence of 10^{-9}M citalopram or 10^{-6}M citalopram. Aldosterone secretion into the medium was measured by RIA. Data from 4 separate experiments, with triplicate incubations performed in each, were combined and expressed as mean±SEM.

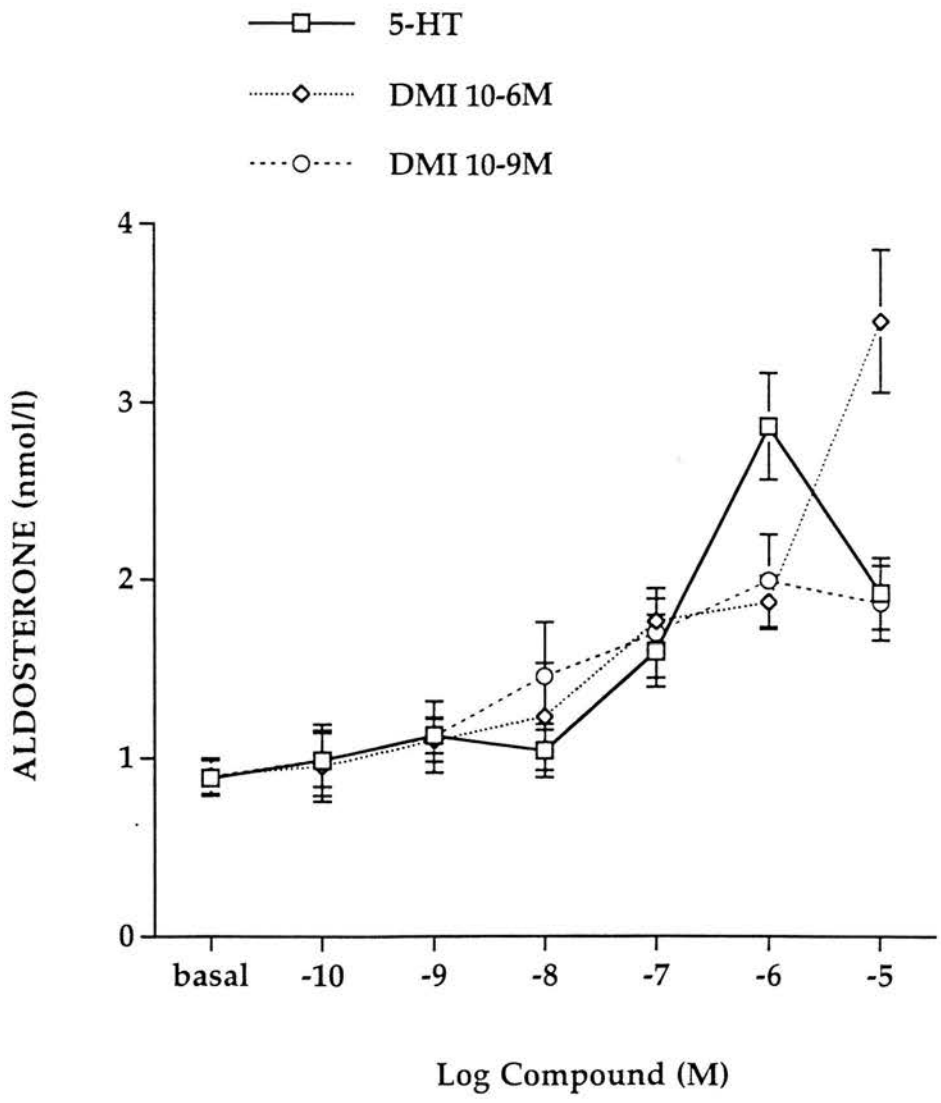


Figure 6.3. *Dose-dependent increase in aldosterone secretion from isolated ZG cells in response to increasing concentrations of 5-HT, in the presence and absence of DMI 10^{-6}M and DMI 10^{-9}M.* Isolated rat ZG cells were incubated for 1 hour at 37°C with increasing concentrations of 5-HT alone or 5-HT in the presence of 10^{-9}M DMI or 10^{-6}M DMI. Aldosterone secretion into the medium was measured by RIA. Data from 4 separate experiments, with triplicate incubations performed in each, were combined and expressed as mean±SEM.

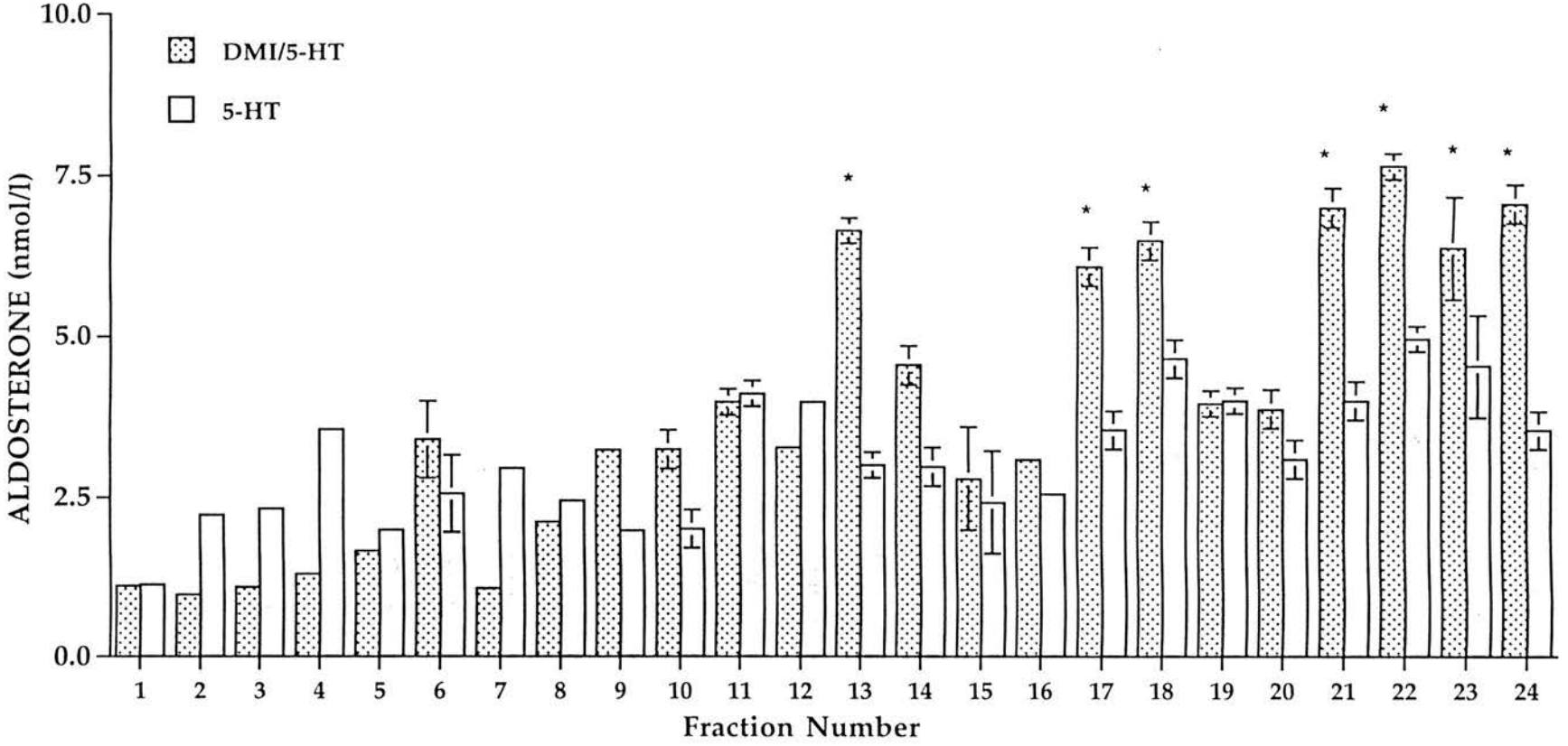


Figure 6.4. *Dose-dependent increase in aldosterone secretion from superfused whole capsular tissue in response to increasing concentrations of 5-HT, in the presence and absence of DMI 10^{-9}M.* Whole adrenal capsules were superfused with increasing concentrations of 5-HT alone or 5-HT in the presence of 10^{-9}M DMI. Perfusate was collected every 5 minutes for 120 minutes. Perfusate sample numbers :- 1-4 = basal, 5-6 = 5-HT10^{-9}M +/- DMI10^{-9}M, 7-8 = washout, 9-10= 5-HT10^{-8}M +/- DMI10^{-9}M, 11-12 = washout, 13-14 = 5-HT10^{-7}M +/- DMI10^{-9}M, 15-16 = washout, 17-18 = 5-HT10^{-6}M +/- DMI10^{-9}M, 19-20 = washout, 21-22 = 5-HT10^{-5}M +/- DMI10^{-9}M, 23-24 = washout. Aldosterone secretion into the medium was measured by RIA. Data from 4 separate experiments, with triplicate incubations performed in each, were combined and expressed

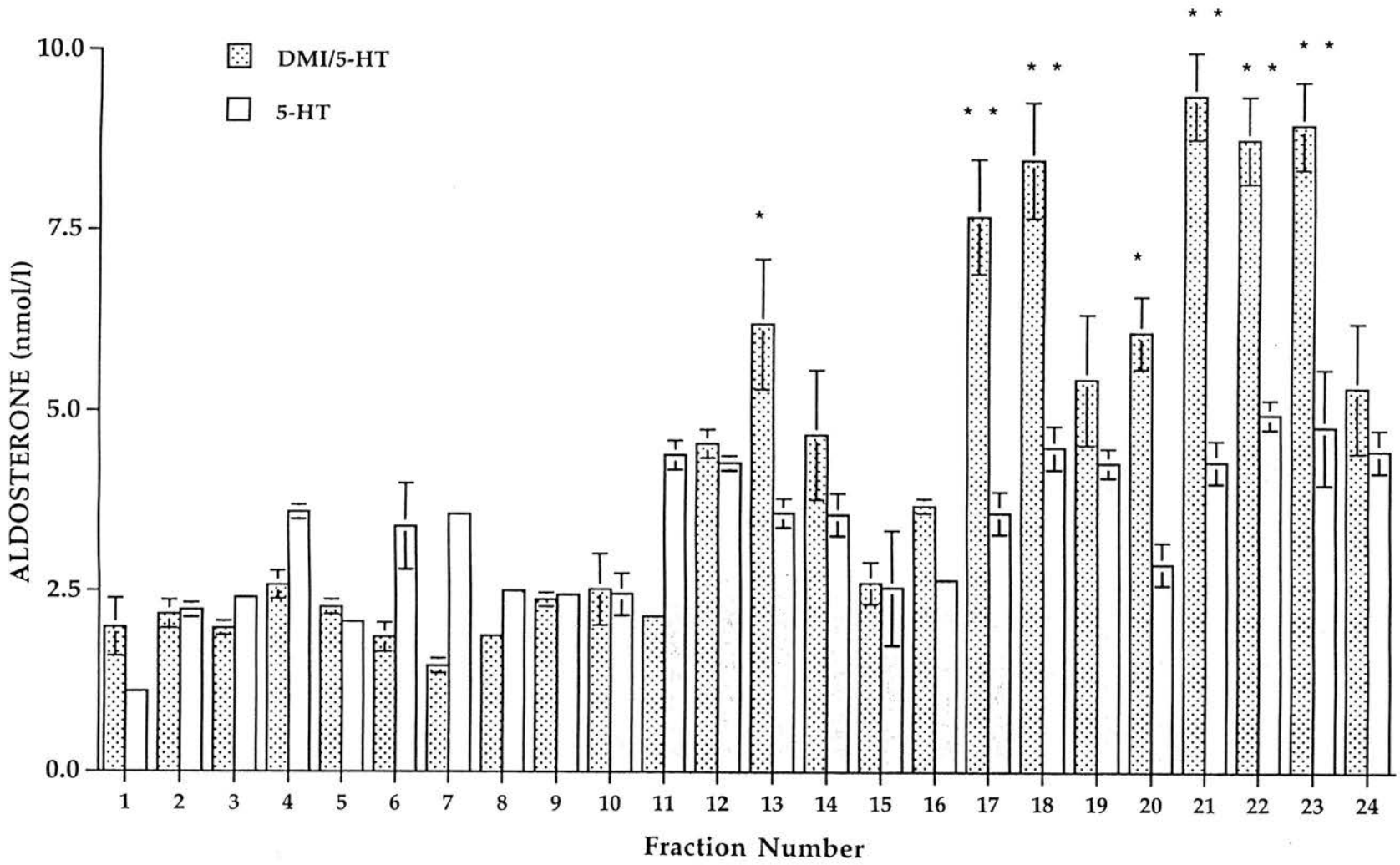


Figure 6.5. *Dose-dependent increase in aldosterone secretion from superfused whole capsular tissue in response to increasing concentrations of 5-HT, in the presence and absence of DMI 10^{-6}M.* Whole adrenal capsules were superfused with increasing concentrations of 5-HT alone or 5-HT in the presence of 10^{-6}M DMI. Perfusate was collected every 5 minutes for 120 minutes. Perfusate sample numbers :- 1-4 = basal, 5-6 = 5-HT10^{-9}M +/- DMI10^{-6}M, 7-8 = washout, 9-10= 5-HT10^{-8}M +/- DMI10^{-6}M, 11-12 = washout, 13-14 = 5-HT10^{-7}M +/- DMI10^{-6}M, 15-16 = washout, 17-18 = 5-HT10^{-6}M +/- DMI10^{-6}M, 19-20 = washout, 21-22 = 5-HT10^{-5}M +/- DMI10^{-6}M, 23-24 = washout. Aldosterone secretion into the medium was measured by RIA. Data from 4 separate experiments, with triplicate incubations performed in each, were combined and expressed

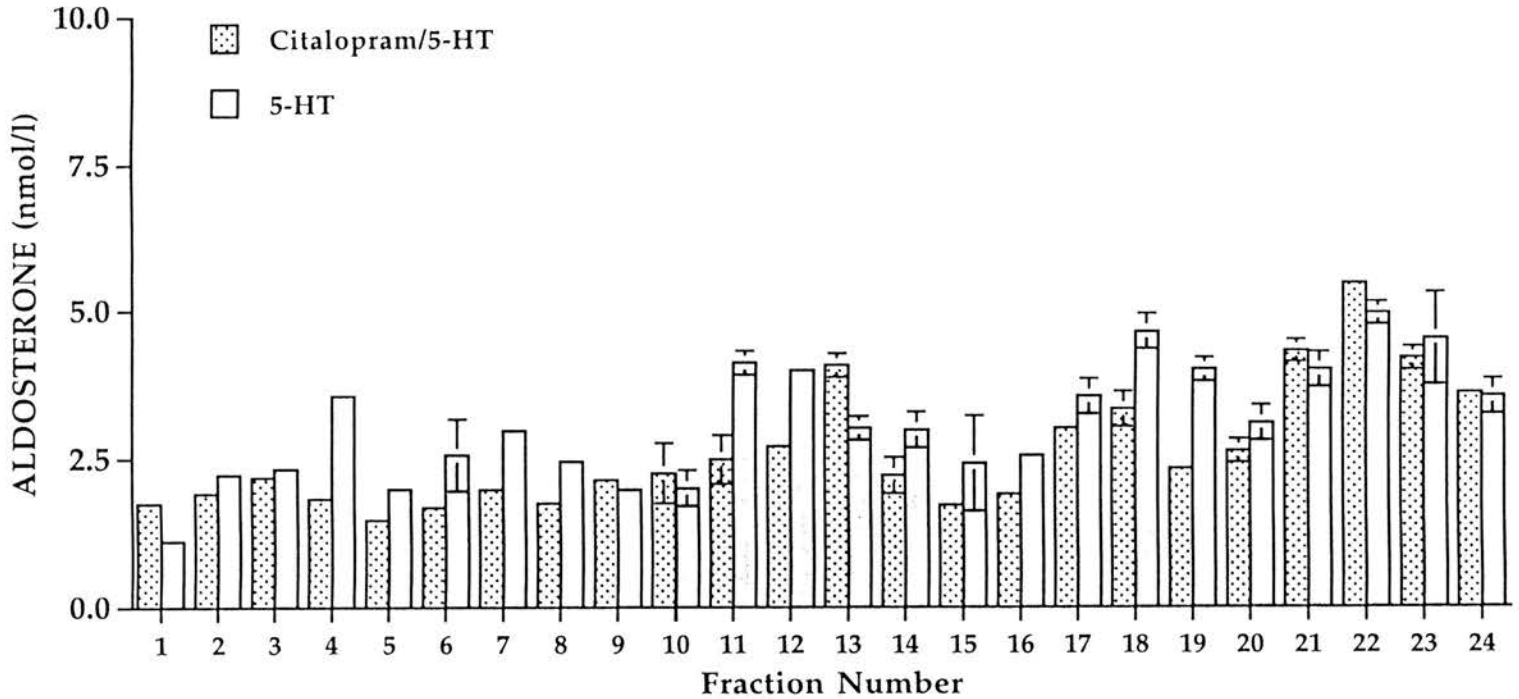


Figure 6.6. *Dose-dependent increase in aldosterone secretion from superfused whole capsular tissue in response to increasing concentrations of 5-HT, in the presence and absence of citalopram 10^{-9}M.* Whole adrenal capsules were superfused with increasing concentrations of 5-HT alone or 5-HT in the presence of 10^{-9}M citalopram. Perfusate was collected every 5 minutes for 120 minutes. Perfusate sample numbers :- 1-4 = basal, 5-6 = 5-HT10^{-9}M +/- citalopram10^{-9}M, 7-8 = washout, 9-10= 5-HT10^{-8}M +/- citalopram10^{-9}M, 11-12 = washout, 13-14 = 5-HT10^{-7}M +/- citalopram10^{-9}M, 15-16 = washout, 17-18 = 5-HT10^{-6}M +/- citalopram10^{-9}M, 19-20 = washout, 21-22 = 5-HT10^{-5}M +/- citalopram10^{-9}M, 23-24 = washout. Aldosterone secretion into the medium was measured by RIA. Data from 4 separate experiments, with triplicate incubations performed in each, were combined and expressed as mean±SEM.

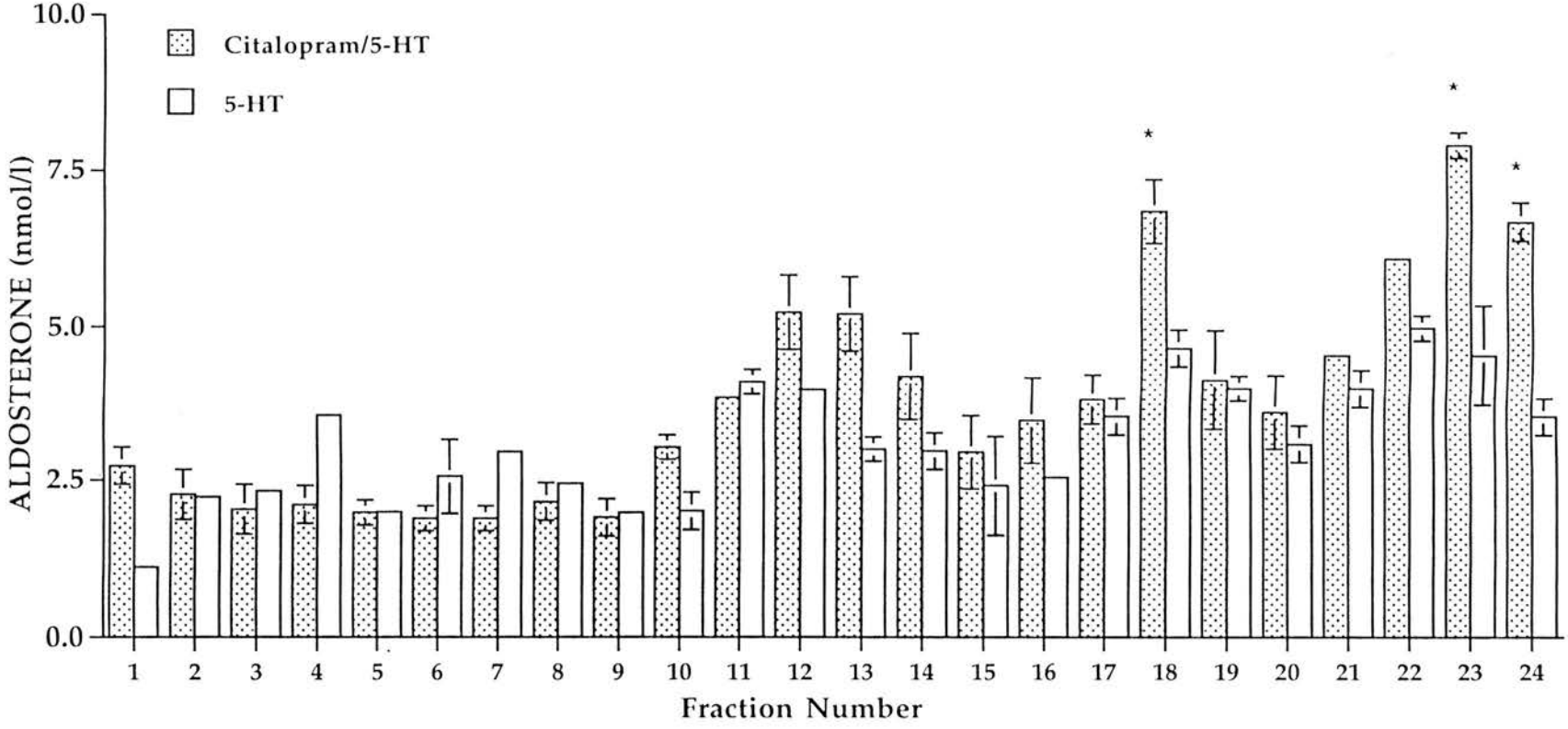
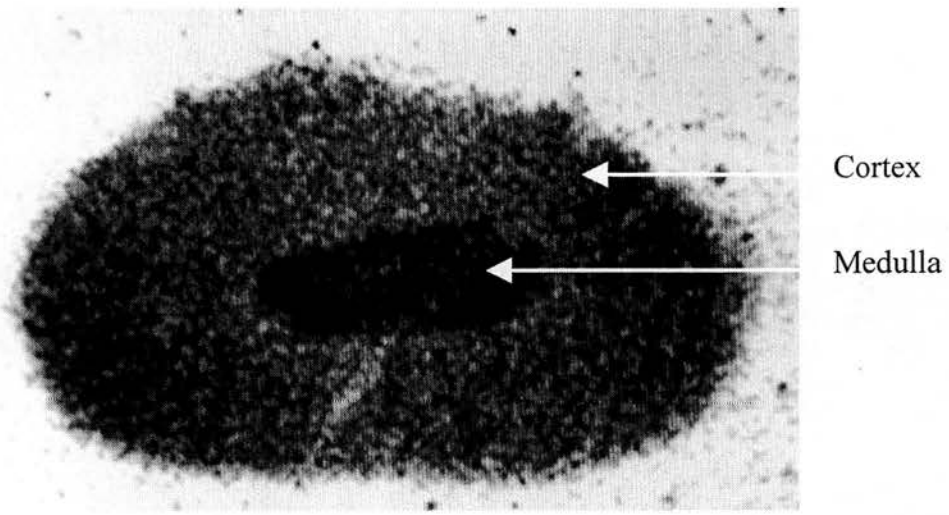


Figure 6.7. *Dose-dependent increase in aldosterone secretion from superfused whole capsular tissue in response to increasing concentrations of 5-HT, in the presence and absence of citalopram 10^{-6}M.* Whole adrenal capsules were superfused with increasing concentrations of 5-HT alone or 5-HT in the presence of 10^{-6}M citalopram. Perfusate was collected every 5 minutes for 120 minutes. Perfusate sample numbers :- 1-4 = basal, 5-6 = 5-HT10^{-9}M +/- citalopram10^{-6}M, 7-8 = washout, 9-10= 5-HT10^{-8}M +/- citalopram10^{-6}M, 11-12 = washout, 13-14 = 5-HT10^{-7}M +/- citalopram10^{-6}M, 15-16 = washout, 17-18 = 5-HT10^{-6}M +/- citalopram10^{-6}M, 19-20 = washout, 21-22 = 5-HT10^{-5}M +/- citalopram10^{-6}M, 23-24 = washout. Aldosterone secretion into the medium was measured by RIA. Data from 4 separate experiments, with triplicate incubations performed in each, were combined and expressed as mean±SEM. *$p<0.05$ compared to

A: Total Binding



B: Non Specific Binding

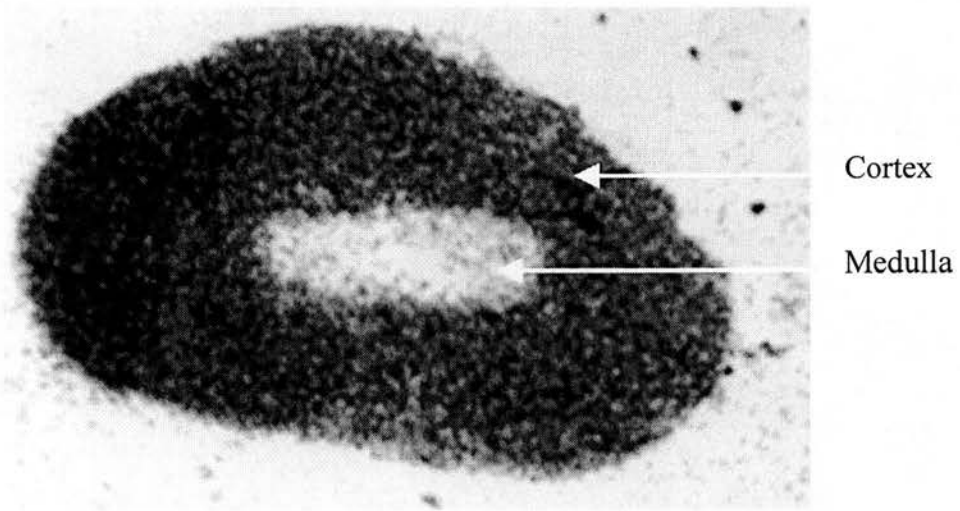


Figure 6.8. : Representative [^{3}H]paroxetine autoradiographs from adjacent cryostat sections of whole rat adrenal gland. Total binding was defined using 250pM [^{3}H]paroxetine (A) and non-specific binding was defined using 250pM [^{3}H]paroxetine in the presence of 4μM citalopram (B). Binding was evident within the medulla (220±20 fmol/mg of tissue), and no binding was evident within the adrenal cortex (7±6 fmol/mg of tissue) (n=4).

Figure 6.9 (a): Sections through the rat adrenal gland stained with the antibody S-240-KLH at magnification x80 and x200.

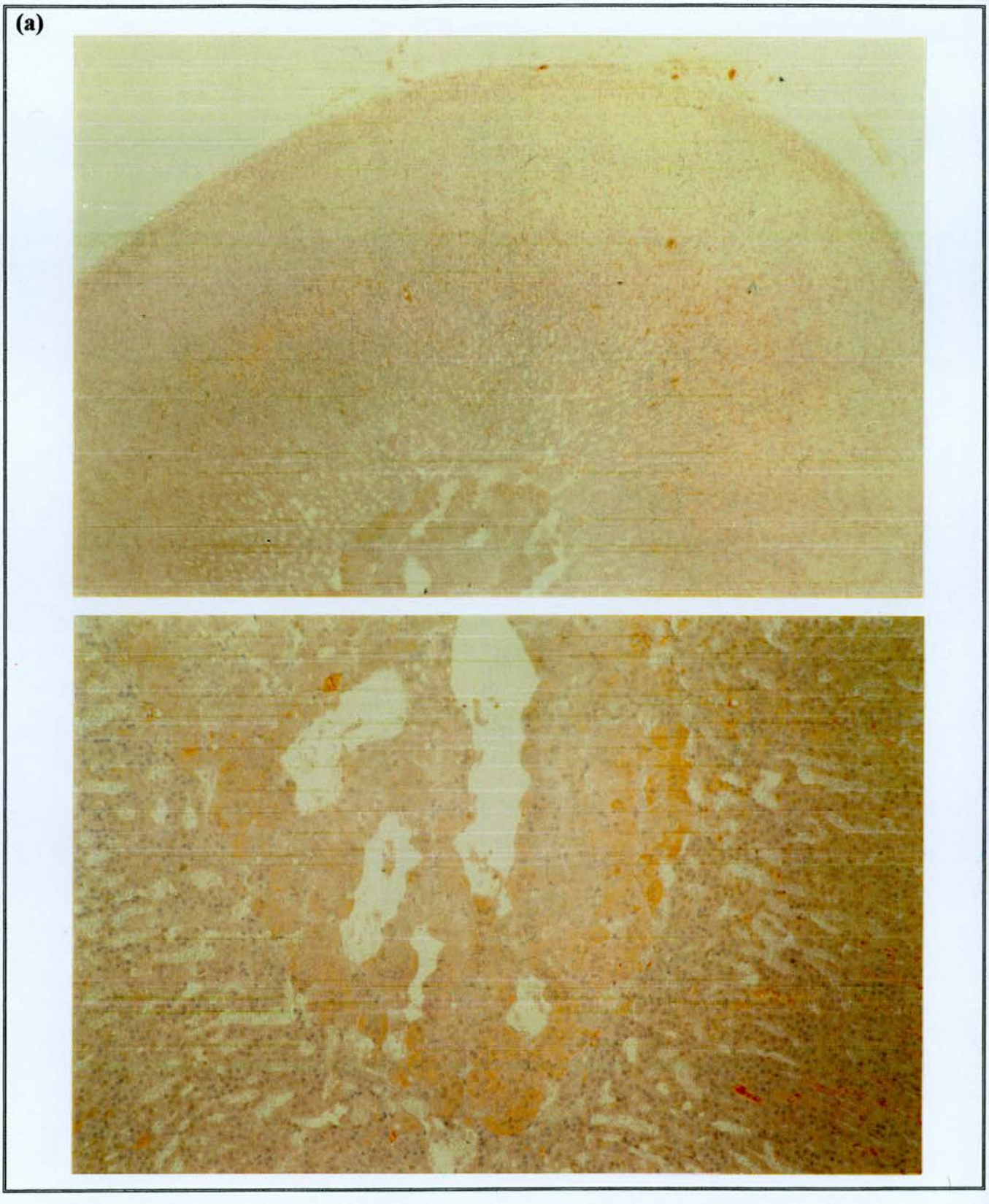


Figure 6.9 (a): Sections through the rat adrenal gland stained with the antibody S-240-KLH at magnification x80 and x200.

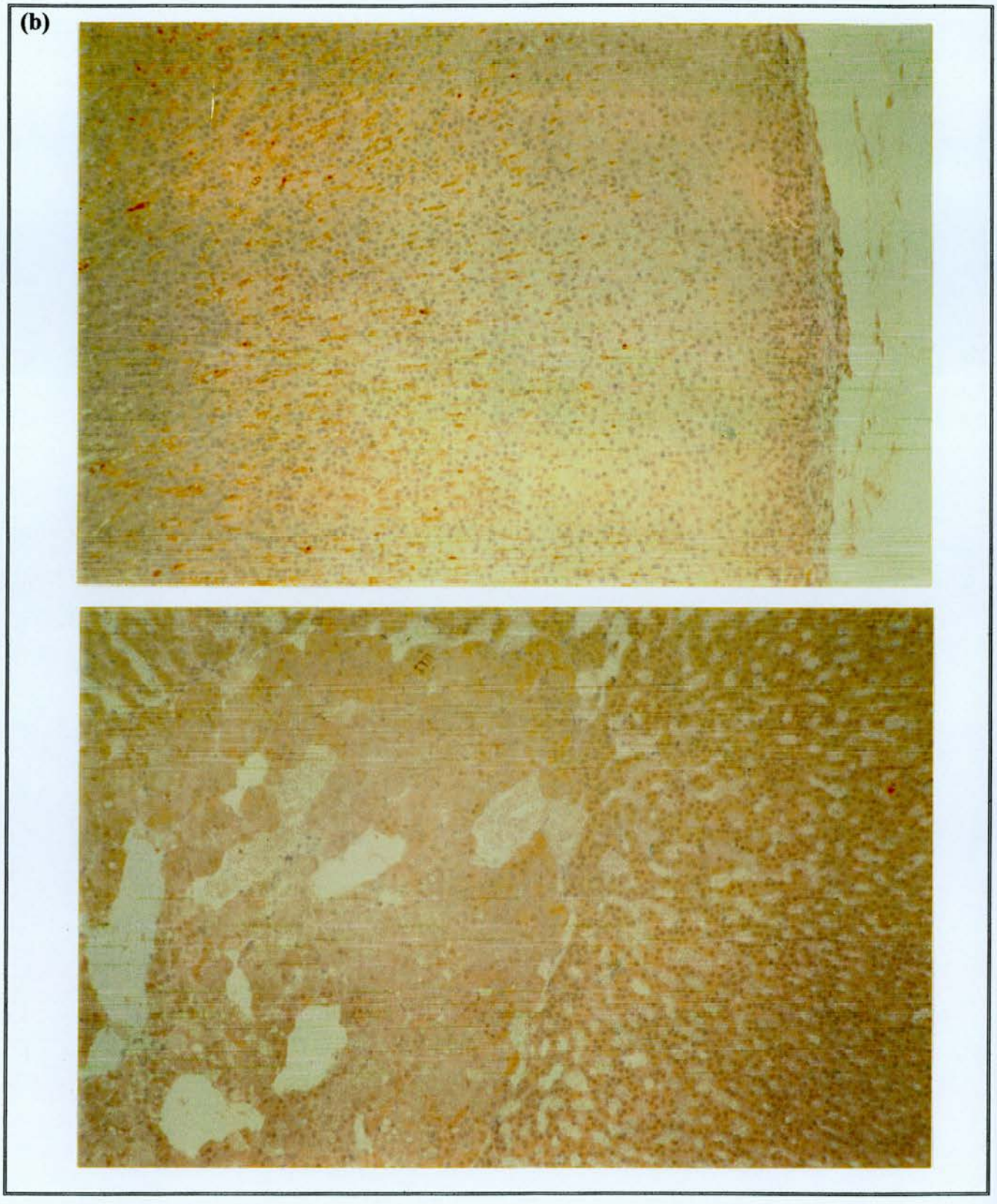


Figure 6.9 (b): Sections through the rat adrenal gland stained with the antibody S-387-KLH at magnification x80 and x200.

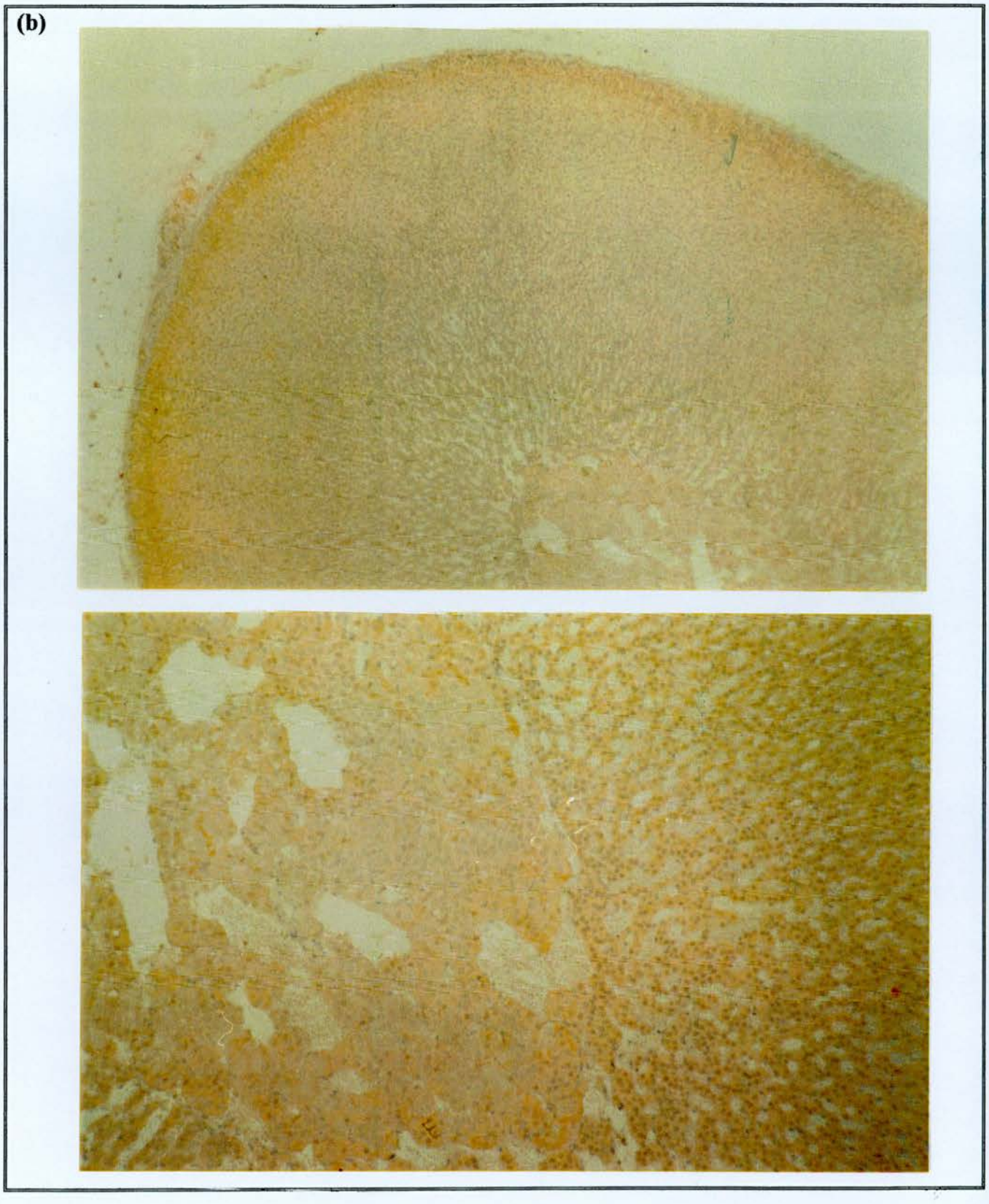


Figure 6.9 (b): Sections through the rat adrenal gland stained with the antibody S-387-KLH at magnification x80 and x200.

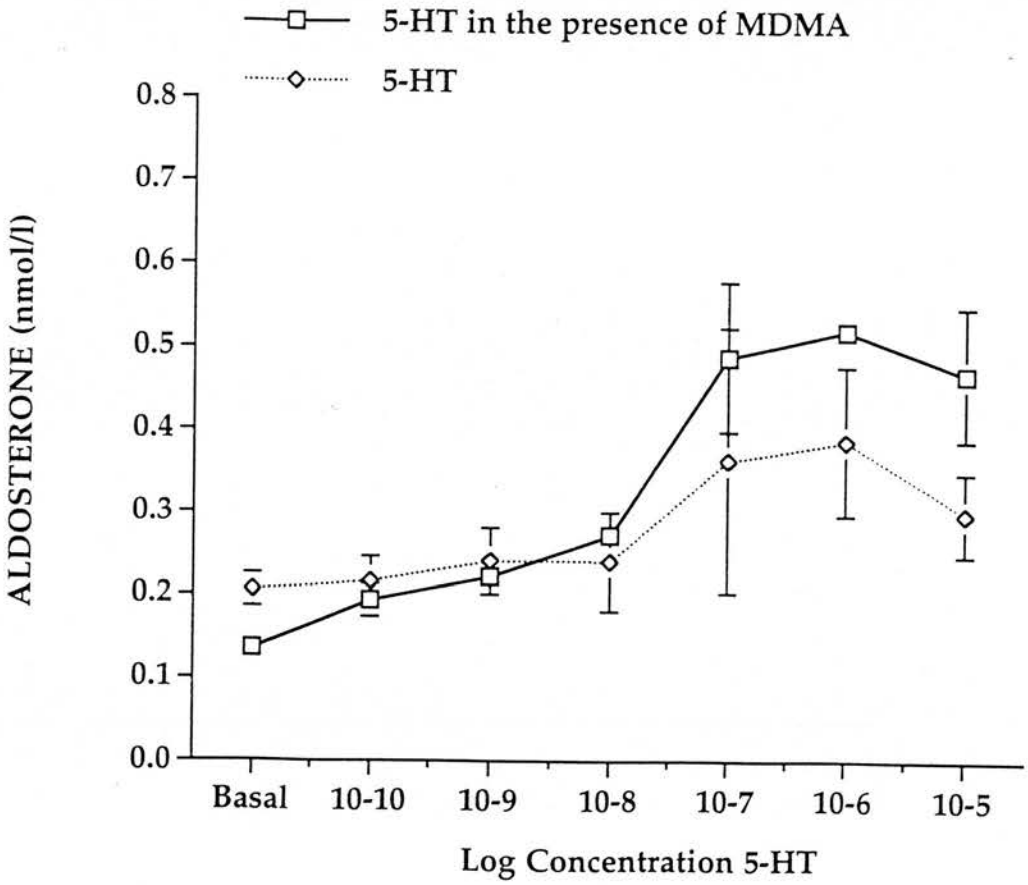


Figure 6.10. *Dose-dependent increase in aldosterone secretion from isolated ZG cells in response to increasing concentrations of 5-HT, in the presence and absence of MDMA 1 μM.* Isolated ZG cells were incubated for 1 hour at 37°C with increasing concentrations of 5-HT alone or 5-HT in the presence of MDMA 1μM. Aldosterone secretion into the medium was measured by RIA. Data from 4 separate experiments, with triplicate incubations performed in each, were combined and expressed as mean±SEM

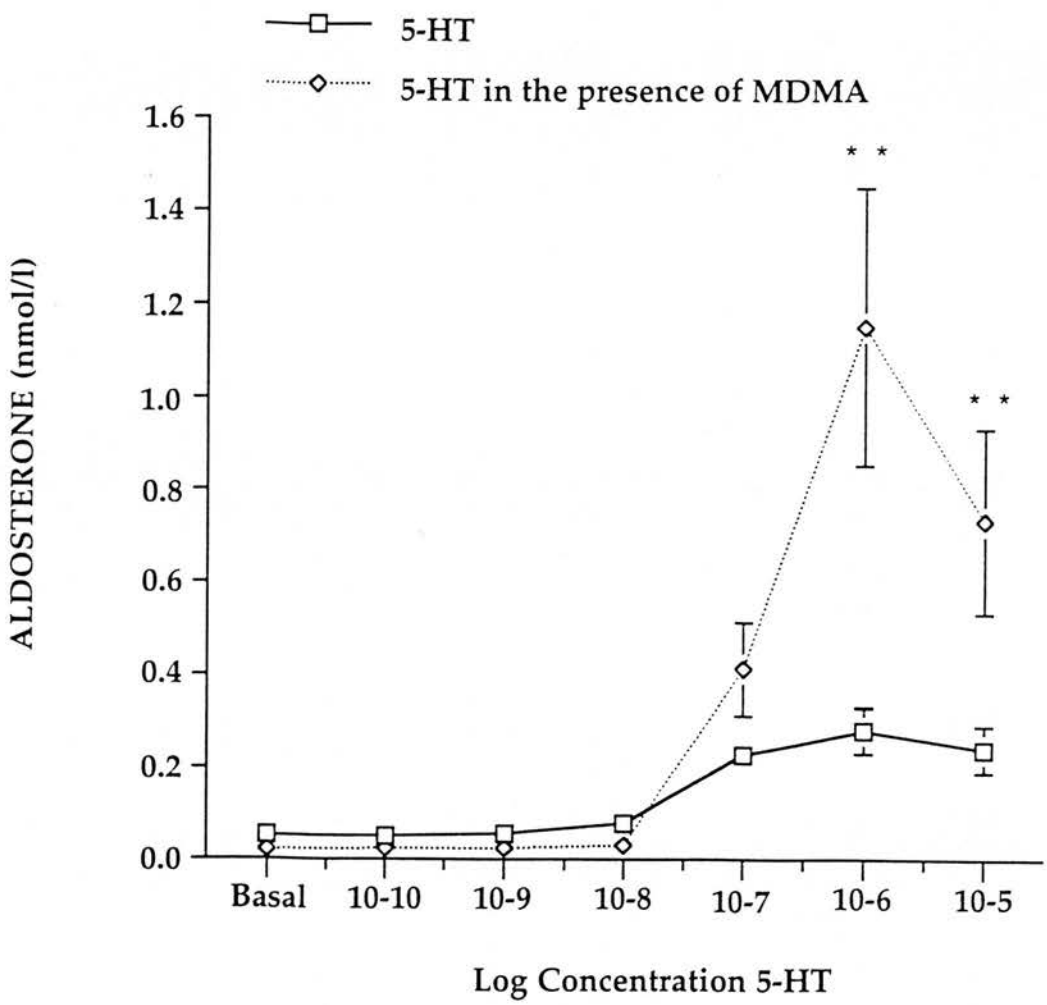


Figure 6.11. *Dose-dependent increase in aldosterone secretion from isolated ZG cells in response to increasing concentrations of 5-HT, in the presence and absence of MDMA 10μM.* Isolated ZG cells were incubated for 1 hour at 37°C with increasing concentrations of 5-HT alone or 5-HT in the presence of MDMA 10μM. Aldosterone secretion into the medium was measured by RIA. Data from 4 separate experiments, with triplicate incubations performed in each, were combined and expressed as mean±SEM. **$p<0.01$ compared to basal levels.

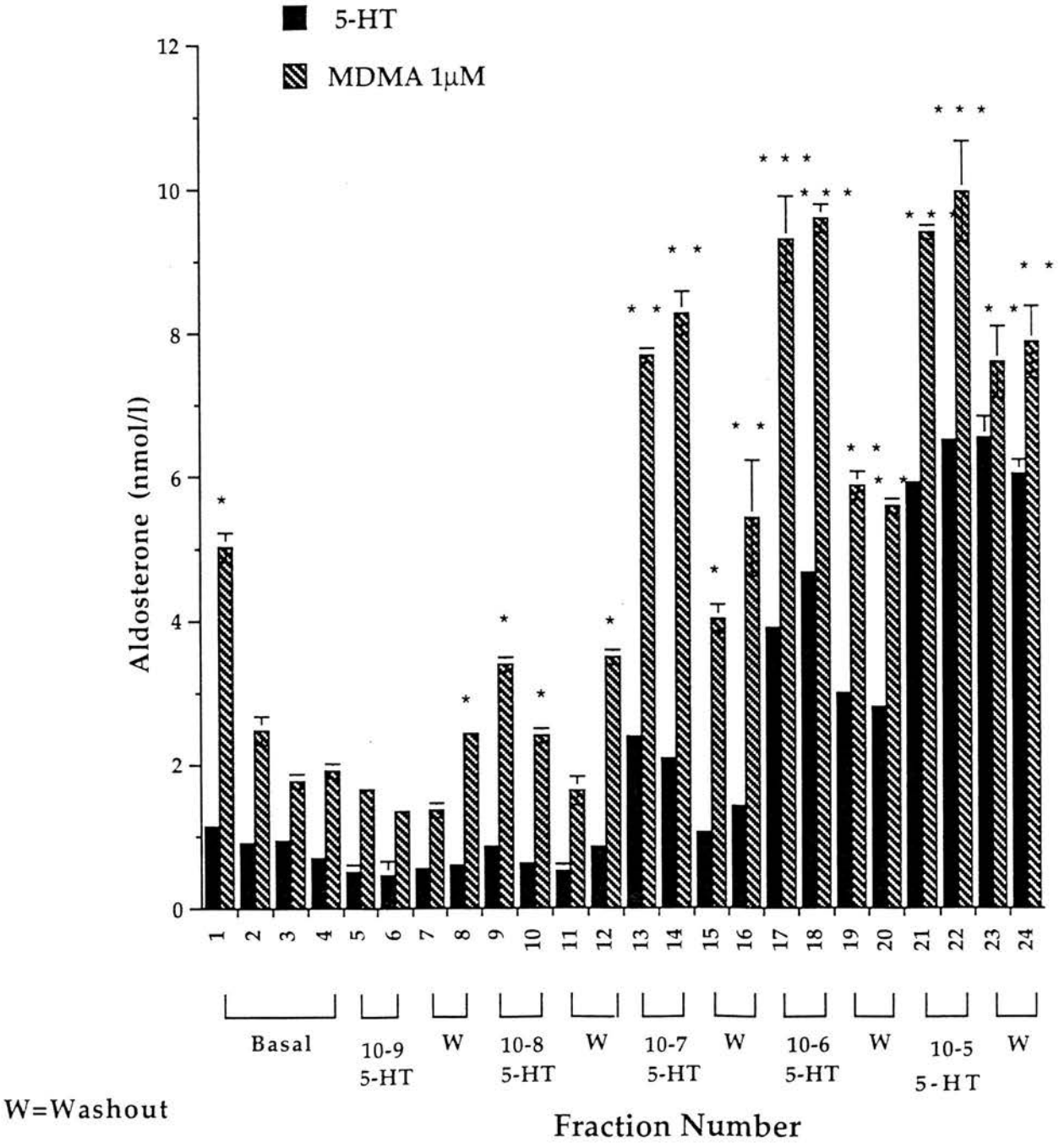


Figure 6.12. *Dose-dependent increase in aldosterone secretion from superfused whole capsular tissue in response to increasing concentrations of 5-HT, in the presence and absence of MDMA 1μM.* Whole adrenal capsules were superfused with increasing concentrations of 5-HT alone or 5-HT in the presence of MDMA 1μM. Perfusate was collected every 5 minutes for 120 minutes. Perfusate sample numbers :- 1-4 = basal, 5-6 = 5-HT10^{-9}M +/- MDMA, 7-8 = washout, 9-10= 5-HT10^{-8}M +/- MDMA, 11-12 = washout, 13-14 = 5-HT10^{-7}M +/- MDMA, 15-16 = washout, 17-18 = 5-HT10^{-6}M +/- MDMA, 19-20 = washout, 21-22 = 5-HT10^{-5}M +/- MDMA, 23-24 = washout. Aldosterone secretion into the medium was measured by RIA. Data from 4 separate experiments, with triplicate incubations performed in each, were combined and expressed as mean±SEM. *$p<0.05$, **$p<0.01$ and ***$p<0.001$ compared to basal levels.

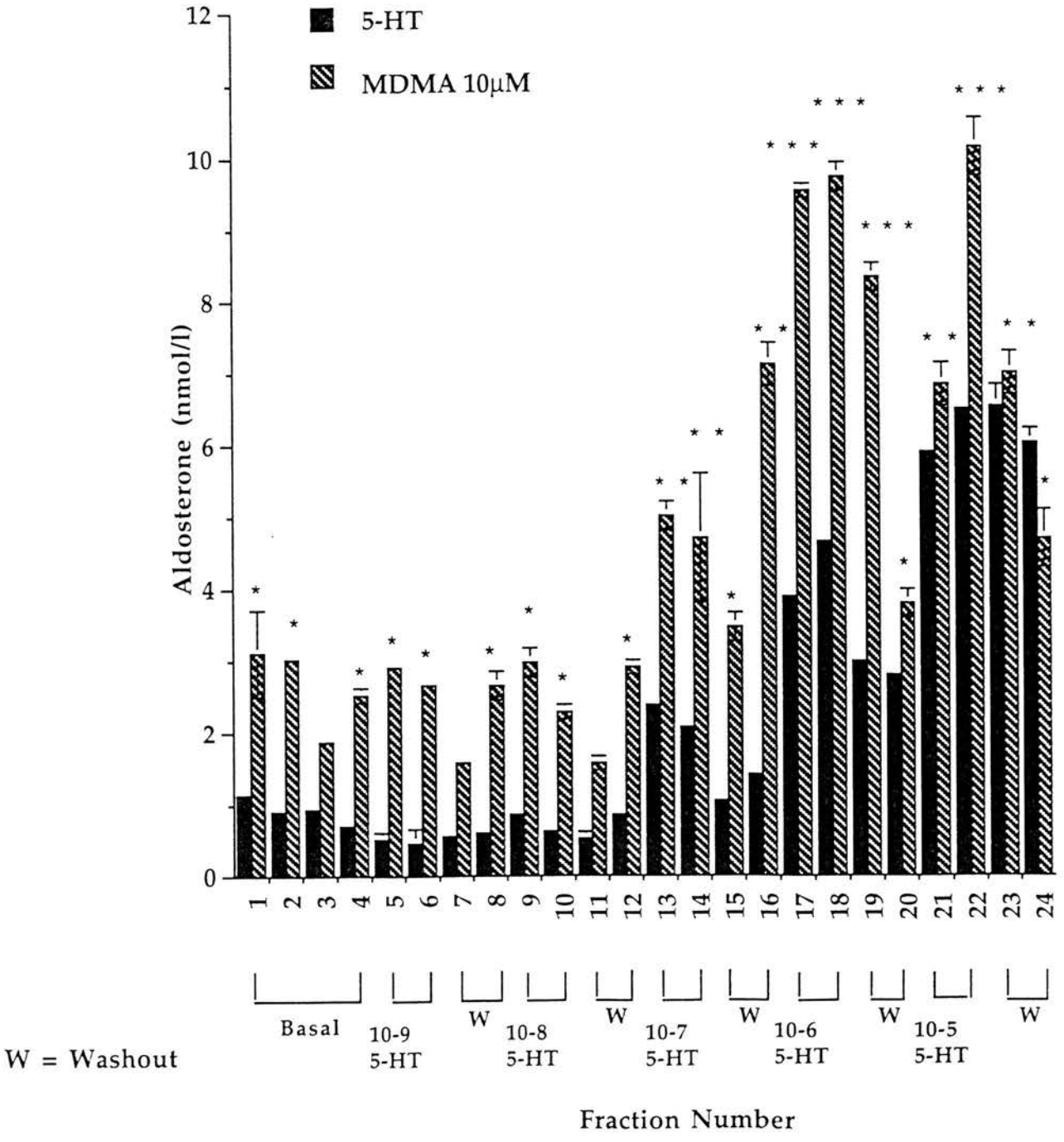


Figure 6.13. *Dose-dependent increase in aldosterone secretion from superfused whole capsular tissue in response to increasing concentrations of 5-HT, in the presence and absence of MDMA 10 μM.* Whole adrenal capsules were superfused with increasing concentrations of 5-HT alone or 5-HT in the presence of MDMA 10μM. Perfusate was collected every 5 minutes for 120 minutes. Perfusate sample numbers :- 1-4 = basal, 5-6 = 5-HT10^{-9}M +/- MDMA, 7-8 = washout, 9-10= 5-HT10^{-8}M +/- MDMA, 11-12 = washout, 13-14 = 5-HT10^{-7}M +/- MDMA, 15-16 = washout, 17-18 = 5-HT10^{-6}M +/- MDMA, 19-20 = washout, 21-22 = 5-HT10^{-5}M +/- MDMA, 23-24 = washout. Aldosterone secretion into the medium was measured by RIA. Data from 4 separate experiments, with triplicate incubations performed in each, were combined and expressed as mean±SEM. *$p<0.05$, **$p<0.01$ and ***$p<0.001$ compared to basal levels.

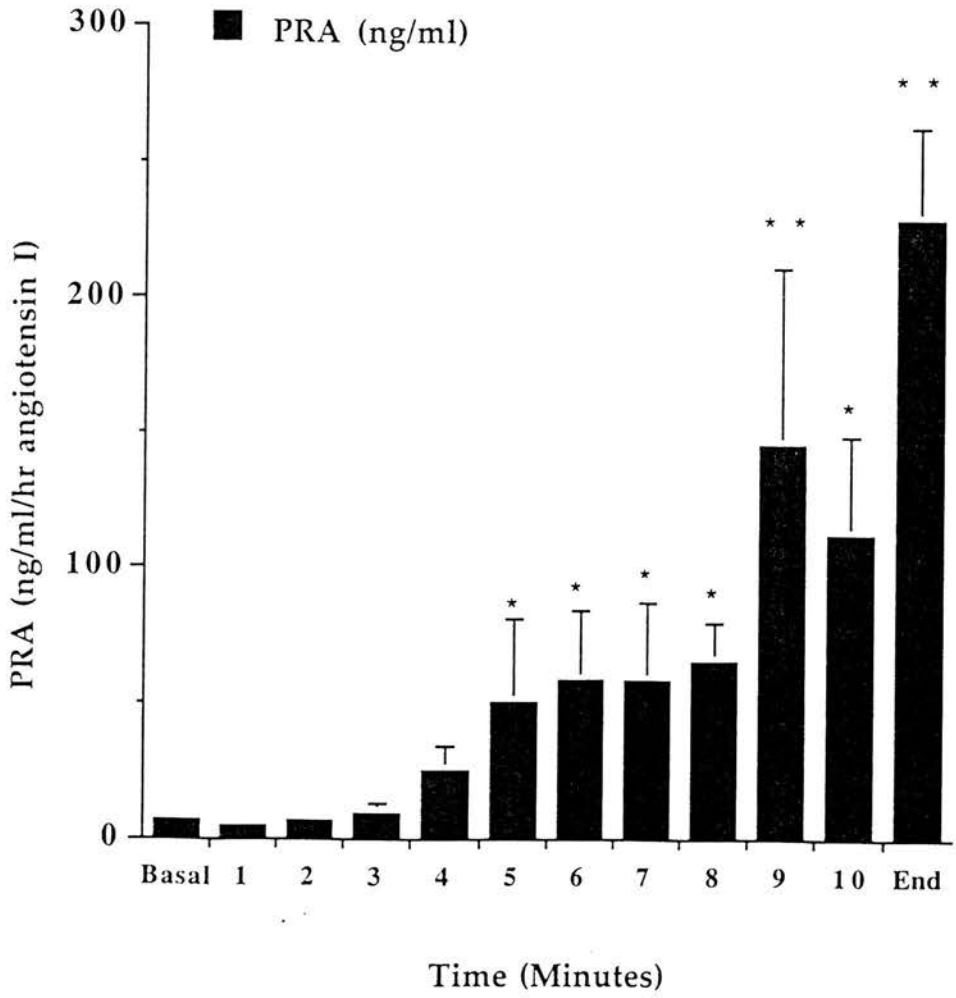


Figure 6.14. (a) *Depicts plasma aldosterone levels in rats after in vivo administration of MDMA.* Values are mean±SEM, n=12. *p<0.05 and **p<0.01 compared with basal levels.

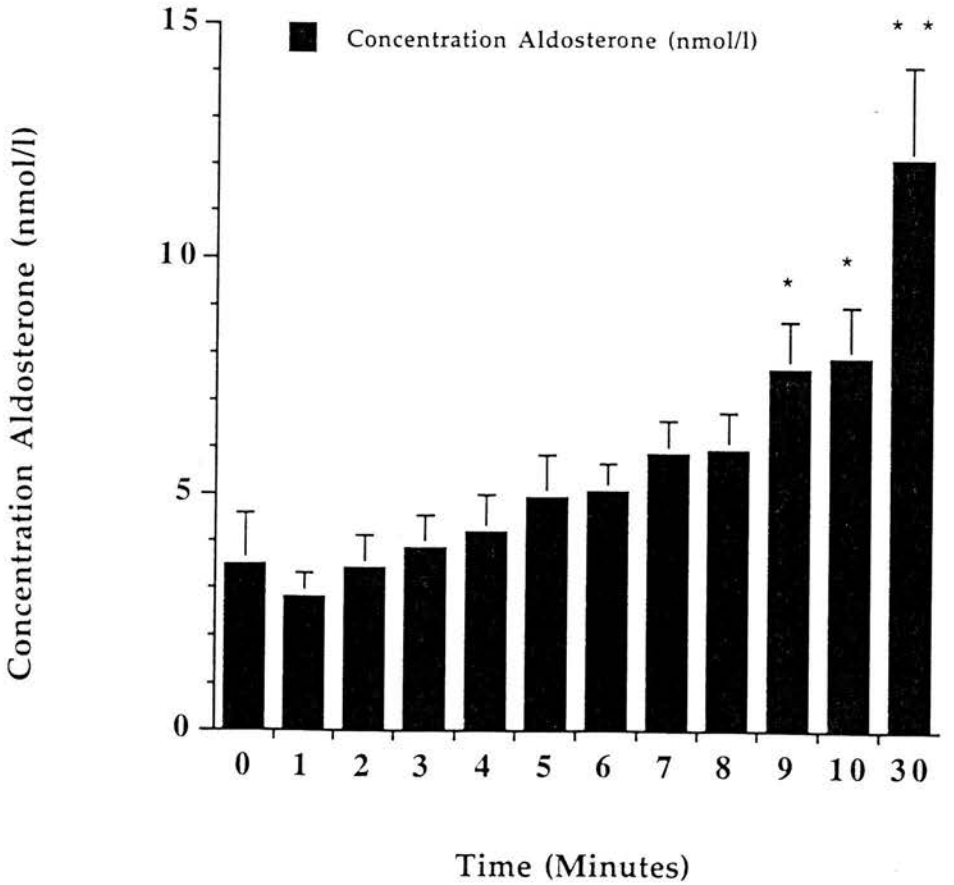


Figure 6.14. (b) *Depicts plasma renin levels in rats after in vivo administration of MDMA.* Values are mean±SEM, n=12. *p<0.05 and **p<0.01 compared with basal levels.

Figure 6.14 A) represents the *in vivo* results for plasma aldosterone in rats after injection with MDMA (n=12). Basal aldosterone levels in the control animals was 3.114 ± 2.1 mmol/l and did not change significantly during the time course of the experiment. In MDMA treated animals basal aldosterone levels were 3.490 ± 1.1 mmol/l, increasing with time to 12.0 ± 2 mmol/l after 30 minutes ($p<0.01$). Basal PRA was 6.8 ± 1.65 ng/ml/hr in the MDMA treated animals increasing to 228.5 ± 34.1 ng/ml/hr after 30 minutes ($p<0.01$) (Figure 6.14B). There were no significant differences in physiological status between the two groups of rats before treatment, or in the control group after injection with saline. Following injection with MDMA, a transient hypertension (MABP in the range 160-190mmHg), rapid in onset and lasting approximatley 1 minute was evident resolving around 2 minutes after injection (MABP 130 ± 8mmHg). This rise in blood pressure coincided with a period of intense motor activity similar to the symptoms seen in the "5-HTergic syndrome". A slight rise in rectal temperature was observed in the MDMA treated animals, although this was not statistically significant.

Preliminary western blot analysis of rat adrenal tissue located the transporter protein within medullary/fasciculata cell pellet tissue, but not within capsular tissue cell pellets. The western blot analysis was only carried out once, due to time limitations (data not shown).

6.4. Discussion.

Many biochemical, physiological and molecular biology experiments support a role for 5-HT within the adrenal gland. The study discussed in Chapter 5 gives one hypothesis for the local source for 5-HT within the rat adrenal cortex. Another hypothesis as mentioned before involves the 5-HT localized within rat chromaffin granules. These

granules also contain large quantities of noradrenaline and adrenaline and are typically associated with stress-induced release of these amines into the circulation (Holzwarth & Brownfield, 1985). It is estimated that as much as 8μM of 5-HT is stored within these granules, and, once released, can elevate extracellular 5-HT above the nanomolar concentrations previously used in *in vitro* studies (Boess & Martin, 1994). So, 5-HT may be transported across the plasma membrane from plasma sources and further concentrated in secretory granules prior to release within the adrenal gland, analogous to mechanisms for platelet 5-HT storage (Gillis & Pitt, 1982).

The results of this study have confirmed the presence of the 5-HT transporter within the medulla of the rat adrenal gland. However from the immunostaining data there would appear to be 5-HT transporter protein within the cortex, and this observation is given credence via the pharmacological studies which show elevated aldosterone secretion from the adrenal capsule and isolated ZG cells when 5-HT is in the presence of citalopram, DMI and MDMA. One anomoly between the isolated cell studies and the whole capsular studies was the very reduced effect seen in the isolated cells. In fact in some experiments no effect of citalopram, DMI and MDMA was seen not only in the isolated cells but also in whole capsular tissue. An explanation for the reduced effect seen in the isolated cell preparation would be the collagenase digestion, which may have destroyed any transporter protein present, or ruptured membranes, leading to a reduced effect. Another more plausible explanation is that the results seen within the cortex are due to the presence of trapped platelets and/or mast cells which contain transporter protein and could be responsible for the effect seen within the rat cortex. As no other group has seen any evidence for the 5-HT transporter within the cortex, this would seem a likely reason for the results reported in this study. Another plausible hypotheses would be the existence of cortico-medullary interactions. Indeed the dark

staining evident with antibody S-387-KLH, was within the cortex associated with the vasculature, and this could be linked to the rays of medullary tissue reported to extend across the cortex following the connective fibres of the large adrenal vein in the rat (Gallo-Payet *et al.*, 1987). Schroeter *et al.*, (1997), reported the existence of the 5-HT transporter within the rat adrenal gland associated with chromaffin granules, more importantly, they noticed intense 5-HT transporter activity within the chromaffin cells on the cortico-medullary border. Co-localisation of the two cell types has been reported, with medullary products exerting effects on the cortex (Bornstein *et al.*, 1992). So the results seen in this study can be explained by the existence of these cortico-medullary interactions. The close proximity of the chromaffin cells containing the transporter as reported by Schroeter *et al.*, (1997), to the steroid synthesizing cells of the cortex, is consistent with a paracrine interaction between the cell types mediated by 5-HT. These chromaffin cells are well positioned to release 5-HT onto the receptor sites within the cortex and modulate corticosteroid release (Lefebvre *et al.*, 1992). In fact since corticosteroids regulate catecholamine release, medullary 5-HT may be released as part of a coordinated feedback loop to ensure integrated function of the adrenal gland (Schubert *et al.*, 1980).

The *in vitro* cell work and superfusion studies described in this chapter provide initial evidence for a role of the 5-HT transporter within the rat adrenal cortex. Further studies are required to fully characterise a role for the protein within the cortex. Is the transporter modulated by salt intake, where is it localised within the ZG? Initial studies carried out with increasing concentrations of ZG cells in incubations with 5-HT, found an increase in aldosterone output in the presence of both uptake inhibitors, although this was not significantly different from 5-HT incubations as controls. The existence of other transporter proteins may have an effect on aldosterone output within the ZG. In

fact, the vesicular transporters that package catecholamines for release also transport 5-HT with high affinity (approx. 8μM), and on granule fusion enough 5-HT could be present within the cortex to produce an increase in aldosterone secretion (Schroeter, *et al.*, 1997).

The results seen from the *in vivo* study resulted in elevated levels of aldosterone and PRA in the rats tested within the plasma. These effects could contribute to the side effects of MDMA such as the stimulation of thirst, the elevation of blood pressure and the "5-HT syndrome". They may alternatively be secondary responses related to acute hypovolaemia and hyponatraemia induced by MDMA. During the study 3 of the rats dies before the 30 minutes experimentation time, an alarming statistic. One interesting study would be to administer a 5-HT antagonist along with the MDMA and measure PRA and aldosterone levels in blood.

MDMA was first synthesised 80 years ago, and is now one of the most commonly used illegal recreational drugs of abuse. Structurally similar to amphetamine and mescaline MDMA has both stimulant and psychotomimetic activity. In the late 1970's and early 1980's MDMA was used "underground" (FDA-unapproved) as an adjunct to psychotherapy, and so now there is a widely held belief amongst users that it is safe to take, despite a growing number of deaths and severe acute toxicity after ingestion of MDMA. Also there is growing concern of the toxic effects of MDMA, both in long term and short term abuse, on 5-HT nerve terminals.

In animals MDMA produces hyperthermia, which amongst other symptoms form part of the "5-HT Syndrome". This syndrome consists of a series of behavioural changes due to increased 5-HT function, including alterations in cognition and behaviour,

changes in mental status, restlessness, myoclonus, hyperreflexia, tremor, shivering, incoordination, diaphoresis and diarrhoea, other symptoms are changes in function of the autonomic nervous system and in neuromuscular activity. The hyperthermia appears to precipitate other severe clinical problems and the outcome is usually fatal. In humans MDMA abuse has led to increasing accounts of severe toxicity, and in a report from the USA, in 7 fatalities, the pattern of toxicity included hyperthermia, convulsions, disseminated intravascular coagulation, rhabdomyolysis and acute renal failure, toxic symptoms that are similar to those seen in the "5-HT Syndrome". In some reported cases of MDMA abuse patients have been admitted with prolonged neuropsychiatric disorders, convulsions, acute renal failure and hepatotoxicity. Thus ingestion of MDMA, acutely or chronically, can lead to a wide range of unwanted side effects, in some cases death. The MDMA drug culture is associated with raves and all night dance parties. 40 years ago amphetamine aggregation toxicity was first reported in mice. If this was applicable to MDMA toxicity, raves and dance parties produce all the conditions required for induction or enhancement of toxicity i.e. crowded conditions, high ambient temperatures, loud noise and dehydrated subjects. The controlled administration of MDMA in rodents and other animals results in long term neurotoxic decreases in 5-HT content in several brain regions, which reflects neurodegeneration of 5-HT terminals. Some studies have observed 5-HT axons undergoing regenerative sprouting following MDMA injury, although it has not been determined whether they re-establish the original innervation pattern. One study by Fischer *et al.*, (1995), looked at 5-HT innervation patterns in rats and squirrel monkeys lesioned with MDMA 12-18 months previously. 5-HT axon projections were studied neurochemically, autoradiographically and immunocytochemically, and in both species substantial 5-HTergic axonal sprouting was observed after MDMA toxicity. However the pattern was highly abnormal, with distant targets (e.g. dorsal neocortex), remaining

denervated, while some proximal targets (e.g. amygdala, hypothalamus) were reinnervated or hyperinnervated. Such lasting changes may result in a lasting reorganisation of ascending 5-HT axon projections, which may have implications in the abuse of MDMA in humans. Unequivocal data demonstrating that similar changes occur in human brain do not exist, but limited and indirect clinical evidence give cause for concern. In a controlled study in humans (McCann *et al.*, 1994), in which 30 MDMA users and 28 controls were admitted to a controlled inpatient setting for measurement of biological and behavioural indexes of central 5-HT function, concentrations of monoamine metabolites in cerebrospinal fluid, prolactin responses to L-tryptophan, nociceptive responses to ischemic pain, and personality characteristics in which 5-HT has been implicated (i.e. impulsivity and aggression) were all measured in both groups. The subjects with a history of MDMA abuse had lower levels of CSF 5-HIAA as compared to controls. Levels of prolactin response to L-tryptophan and ischaemic pain response were not significantly different between the groups, however MDMA users had significantly lower scores on personality measures of impulsivity and indirect hostility. The CSF findings suggest that 5-HT neurotoxicity may be a potential complication of MDMA abuse, and the differences observed in personality tests further support the view that 5-HT systems are involved in modulating impulsive and aggressive personality traits. Another attempt to study the potential effects of MDMA abuse in the human brain was to use rhesus monkeys and examine the effects of repeated systemic administration of MDMA (2.5-10 mg/kg twice daily for four days), on selected neurochemical and behavioural measures. After four days CSF concentrations of 5-HIAA and brain concentrations of 5-HT and 5-HIAA were decreased significantly. At the high doses of MDMA a selective decrease in 5-HT uptake sites, reflecting destruction of brain 5-HT terminals, was observed. The monkeys were monitored for fourteen days after high dose ingestion for four days in a

subsequent study, to determine if the changes observed in the previous study were pharmacologic or truly neurotoxic. Throughout the fourteen days CSF 5-HIAA remained significantly decreased as opposed to control animals. At the end of the fourteen days, significant decreases in the concentration of 5-HT, 5-HIAA and 5-HT uptake sites were observed in cerebral cortex and striatum, but not in hypothalamus or spinal cord. Noradrenergic and dopaminergic measures in CSF and brain remained unaffected. The authors suggested from this study that the potent and selective toxicity produced by ingestion of MDMA on a chronic basis seen may also be produced in humans (Insel *et al.*, 1989).

MDMA thus causes wide spread toxicity in various species. Numerous studies in rat brain have revealed that MDMA has potent selective neurotoxic properties on 5-HT neurones. Exposure to MDMA in the rat has resulted in profound decreases in the concentrations of 5-HT and its metabolites, the depletion of 5-HT uptake sites and a marked reduction in the density of 5-HT immunoreactive axon terminals (Battaglia *et al.*, 1987, 1987, 1988). Deficits in 5-HT markers in rat have been shown to last up to six months after ingestion of MDMA (Battaglia *et al.*, 1988). Anatomically in rat brain the effects of MDMA are manifest in the terminal fields of 5-HTergic neurones whilst the cell bodies of the dorsal and median raphe nuclei remain relatively unaffected (Molliver,1987; O'Hearn *et al.*, 1988). However within some terminal fields, most importantly neocortex and hippocampus, the loss of 5-HT fibres is not homogenous, with some subregions of these structures being profoundly affected whilst others are relatively spared (O'Hearn *et al.*, 1988).

But what of the mechanism of action of MDMA. 5-HT transport has been implicated in the mechanism of action of a number of amphetamine derivatives including *p*-

chloroamphetamine and fenfluramine. These indirectly acting sympathomimetic amines exert their effects by releasing endogenous biogenic amines from nerve terminals, and in fact release 5-HT preferentially both *in vivo* and *in vitro*. (Fuller *et al.*, 1965; Schmidt *et al.*, 1987). MDMA (3,4-methylenedioxymethamphetamine; MDMA), as is suggested from its chemical name, is a ring substituted derivative of methamphetamine, and as such exerts effects on monoaminergic systems in brain comparable to those of amphetamines. The 5-HT transporter has been implicated in the mechanism of action of amphetamine based drugs as inhibitors of 5-HT transport block the effect of these drugs on acute 5-HT release and destruction of 5-HTergic terminals (Schmidt *et al.*, 1987; Fuller *et al.*, 1975), suggesting that the 5-HT transporter either mediates the entry of neurotoxic amphetamines, like MDMA, into 5-HTergic terminals or participates in sequelae leading to 5-HT release and depletion or both. Calcium ions are not required for amphetamine induced 5-HT release, suggesting that exocytosis is not involved. The likely mechanism of action is via reversal of the transport systems that normally catalyze accumulation of 5-HT to high levels within the neuron and synaptic vesicle. In addition to releasing 5-HT, amphetamine derivatives also lead to a long-term depletion of 5-HT (Ricuarte *et al.*, 1985), which correlates with morphological damage to 5-HT nerve endings (Molliver & Molliver, 1990). 5-HT release may be only one of many processes contributing to neurotoxicity. Recently evidence has accumulated implicating dopamine release in the action of neurotoxic amphetamines, in fact dopamine receptor blockers prevent MDMA neurotoxicity (Johnson & Nicols, 1991; Schmidt *et al.*, 1985). Thus other potential mechanisms of neurotoxicity involve an action of released dopamine on serotonergic terminals, also dissipation of ion gradients by transporter-mediated amphetamine cycling and unspecified intracellular actions of the amphetamines and their metabolites (Fuller, 1980; Stone *et al.*, 1988; Schmidt *et al.*, 1990; Rudnick & Wall., 1992).

The previous perception of MDMA as a safe drug of abuse has been revised in the light of recent press coverage of deaths linked to MDMA abuse. Uncertainty remains however as to whether the deaths and toxic side effects are solely due to MDMA ingestion, or are due to contaminants in the illicit MDMA bought at raves etc., and also to what the individual has also ingested that evening (i.e. alcohol, speed, cannabis etc.). What is not in question is that in animal studies to date MDMA produces long lasting toxic effects in the brain, but there has been no evidence that any lasting functional defecit in the human brain occurs.

The results from our study show marked changes in aldosterone and PRA levels after MDMA ingestion. However, this can not be linked to effects that may be seen in humans after ingestion of MDMA for example at a rave. Most MDMA tablets are mixed with various other compounds, the actual dose of MDMA ingested ranges from tablet to tablet. Further studies are needed within rat brain, and with differing concentrations of MDMA to enable a clearer picture of what actually happens *in vivo.*

The 5-HT transporters are a major target for antidepressant medications and with the localisation of the transporter within the adrenal cortex, it remains a possibility that modulation of adrenal catecholamine or steroid production may be a property of these agents. Within the CNS, transporter blockade results in elevated 5-HT exposure to receptors and a downregulation of the receptor (Artigas *et al.*, 1996). Further studies are required to determine whether antagonsim of the adrenal 5-HT transporter may result in heightened responsiveness and/or downregulation of adrenal 5-HT receptors, leading to alterations in steroid hormone output.

Soon after its chemical identification the structural similarities between 5-HT and lysergic acid diethylamide (LSD) led to the logical speculation that substances related to 5-HT may cause mental aberrations. Coincidentally, physicians working in sanitariums for tuberculosis noted that iproniazid, an antitubercular drug with monoamine oxidase inhibitory properties, improved mood in many patients. Also in the 1950's, it became evident in patients treated with reserpine, an antihypertensive agent which depletes monoamine stores, depression was an unwanted side effect to therapy. So nearly 50 years ago the first idea that 5-HT was important in psychiatric illnesses was viewed.

Depression is a heterogenous disease state characterised by complex alterations in several CNS neurotransmitter and receptor systems. All antidepressants are thought to act by causing postsynaptic adaptive changes within these systems. Thus, the mechanism of action of selective 5-HT reuptake inhibitors (SSRIs) cannot simply be explained in terms of inhibition of 5-HT reuptake.

Within the CNS the vast majority of 5-HTergic nerve terminals originate in neuronal cell bodies of the raphe nuclei in the brainstem. These 5-HTergic perikarya project in a topographic fashion to neuroanatomically discrete areas, resulting in a diffuse but heterogenous innervation throughout the brain (Azmitia, 1987). 5-HT acts predominantly as an inhibitory neurotransmitter in the CNS. After release from presynaptic 5-HT containing terminals, 5-HT present within the synapse is inactivated by uptake into the presynaptic terminal by the 5-HT transporter. Once taken up, 5-HT may be degraded by MAO to 5-HIAA, or repackaged into secretory vesicles by the vesicular monoamine transporter (Chapter 1).

Specific inhibitors of monoamine transporters have been important experimental tools in elucidating physiological roles of monoaminergic neurons. For example, SSRIs have helped to establish a role of brain 5-HT neurons in influencing some types of behaviour, the choice and amount of food ingested, sleep and endocrine regulation (Fuller *et al.*, 1991). The tricyclic antidepressant drugs have long been thought to owe their therapeutic efficacy to inhibition of monoamine uptake, particularly of 5-HT and noradrenaline. Most of these drugs are relatively non-selective in their uptake inhibition, and also they have high affinity for numerous neurotransmitter receptors, including muscarinic cholinergic receptors and H_1 histaminergic receptors (Richelson & Pfenning, 1984). In recent years, more selective reuptake inhibitors have been developed. These agents are more selective in their specificity of uptake inhibition as well as in their lack of affinity for neurotransmitter receptors. A growing list of structurally diverse SSRIs are now available and are effective antidepressant drugs (Fuller, 1993). Also SSRIs are effective in the treatment of bulimia, obesity and obsessive-compulsive disorder (Fuller *et al.*, 1994). The full range of therapeutic roles for the SSRIs is still to be elucidated. SSRIs that lack affinity for neurotransmitter receptors may have advantages in clinical use over the tricyclics. They lack anticholinergic and other side effects. Does selective inhibition of the transport of one particular monoamine offer clinical advantages in the treatment of depression over the inhibition of transport of more than one monoamine? This remains to be determined. Perhaps in the future it may be possible to identify patients most likely to respond to a 5-HTergic, a dopaminergic or a noradrenergic drug therapy. In addition to mental depression, transport inhibitors may be useful in treating other disease states, with the SSRIs having the most widespread therapeutic use.

Some neurotoxins appear to rely on transporters to gain entry into the cell cyptoplasm where they exert their nerve killing action. The possibility that neurodegenerative diseases such as parkinsonism may be triggered by such a process remains an attractive hypothesis. Studies with non-toxic transport inhibitors, imipramine and fluoxetine, found that they blocked the toxicity of transported toxins in each case (Rudnick *et al.*, 1992). Studies like these hold the possibility of preventative treatment to slow or stop the progress of neurodegenerative diseases such as parkinsonism that might be attributable to such transported neurotoxins. The drug of abuse cocaine exhibits apparent competitive inhibition of transport of dopamine, but is not a transported substrate. Identification of the regions of the dopamine transporter which recognises cocaine but does not interfere with dopamine-recognition sites may allow a drug addiction medication to be found, a molecule that would prevent cocaine binding without interfering with transport function. The new transporter clones not only provide templates for drug screening, they may also act as guides in the discovery of novel neurotransmitters and neuromodulators. With the rapid growth in identified clones, an equal number of 'orphan' clones has been discovered, with no currently identified transport substrates. These 'orphans' may uncover new chemical neuromodulators or new uses for existing compounds. Rapid developments in molecular understanding of transporters are now poised to enable dramatic advances in our understanding of synaptic function and in drug design.

In conclusion, the 5-HT transporter has been localised within the rat adrenal medulla. Staining was also apparent in areas of the cortex, associated with the vasculature. Pharmacological studies with the 5-HT uptake inhibitors, citalopram and DMI, and the drug of abuse MDMA, would support a role of the 5-HT transporter within the cortex, although this needs further clarification. The drug of abuse, MDMA, produced

elevations in aldosterone and PRA levels in the plasma of rats. These may cause the side-effects seen with ingestion of MDMA, or may be secondary to acute hypovolaemia and hyponatraemia. Further studies will include, studies on the uptake of tritiated 5-HT into isolated ZG cells and whole capsular tissue, more extensive immunohistochemical studies and physiological studies within the whole rat to elucidate a role for the transporter within the gland, and its possible role in depression. Sodium loading and depletion studies would be interesting to see if the transporter molecule is regulated within the adrenal by sodium content.

Chapter Seven

General Discussion and Future Studies.

7.1. General Discussion

The main aim of this thesis was to establish a role for 5-HT in the control of aldosterone secretion. 5-HT is well known for its roles as a neurotransmitter and in the blood clotting cascade but the steroidogenic action of 5-HT in the adrenal gland is an area of interest that still needs to be defined.

In attempting to clarify a physiological role for 5-HT in aldosterone secretion, it is necessary to address a few questions. Is 5-HT able to stimulate aldosterone directly? Does the sensitivity of this aldosterone response to 5-HT increase in states of sodium depletion? If 5-HT does influence aldosterone secretion, what is the physiological source of 5-HT reaching the ZG and can this source also be regulated by salt intake? Are there specific receptors for 5-HT within the rat ZG? Does the 5-HT transporter have a role to play in the ZG?

Classically, 5-HT has always been thought to be the major indoleamine involved in the modulation of aldosterone secretion from the zona glomerulosa, a fact that has not been proven. To resolve this, numerous indoleamines, both naturally occurring and synthetic congeners, were studied in isolated rat zona glomerulosa cells for their ability to induce aldosterone secretion, as compared to 5-HT. The rationale behind this study was two fold, firstly to determine how specific 5-HT-stimulated steroidogenesis is in relation to other structure-related indoleamines and to define more clearly the structure-activity requirements for this stimulation in the ZG cells, and secondly to determine if the metabolites, precursors or intermediates of the 5-HT biosynthetic and metabolic pathways could give rise to a steroidogenic response.

Of the compounds tested, 5-methoxytryptamine, tryptamine, N-methyltryptamine, 5-methyltryptamine, 6-methoxytryptamine and 5-hydroxytryptophan stimulated

aldosterone secretion, to various degrees, from the zona glomerulosa. Of those compounds tested that produced stimulation of aldosterone comparable to that produced by 5-HT, cAMP output was also investigated.

The study allowed specific structural requirements for stimulation of aldosterone secretion to be characterised, namely that the 5-hydroxyl grouping was not required for full agonist activity but ring substitutions at other positions compromise agonist activity. The basicity of the terminal amine group is also important in receptor binding. Compounds lacking both a basic terminal amine grouping and an hydroxy, methyl or hydroxymethyl grouping at C_5 lack aldosterone stimulating activity. This study will aid in the search for specific agonists and antagonists, to be used for research into the zona glomerulosa 5-HT receptor. The role of 5-methoxytryptamine, a naturally occurring indoleamine, within the adrenal gland deserves further investigation.

Unlike the cardiovascular system and CNS, where specific receptors have been identified and categorised, specific 5-HT receptors within the rat zona glomerulosa have still to be fully characterised. The second study of this thesis aimed at characterising these receptors by utilising an array of 5-HT agonists and antagonists.

The antagonists ketanserin and mesulergine were found to inhibit 5-HT-induced aldosterone secretion, and the agonists 5-methoxytryptamine and 5-carboxamidotryptamine, produced identical stimulation of aldosterone from rat zona glomerulosa as that produced with 5-HT. This effect was also found to be affected by sodium status.

These results taken together with previous studies and preliminary studies with a 5-HT_7 probe in sections of rat adrenal gland would suggest the presence of a 5-HT_7 receptor

in rat zona glomerulosa, although the presence of other 5-HT receptors can not be ruled out. The presence of the 5-HT_7 receptor in the rat ZG was confirmed by Contesse *et* al., (1999). With the advent of new and more selective antagonist radioligands such as [^{3}H]-SB-2669970 (Thomas *et al.*, 2000), this receptor site may be more fully characterised and the regulation of these receptors within the adrenal cortex may be more fully investigated.

For 5-HT to have a physiological role in aldosterone secretion, a local source of 5-HT would be required. In consideration of this, the role of the enzyme L-AAAD in the local production of 5-HT and dopamine was investigated in rat adrenal zona glomerulosa in animals maintained on varying sodium diets. The presence of L-AAAD was located, via immunohistochemistry, in the zona glomerulosa, zona fasciculata and the medulla. Conversion of 5-HTP to 5-HT and L-DOPA to dopamine was observed in capsular and medullary tissue preparations; this was inhibited by carbidopa. 5-HTP significantly stimulated aldosterone secretion, from capsular tissue. This effect was more apparent in animals on a low salt diet and less apparent in high salt diet animals than animals maintained on a normal salt diet. The opposite was true for studies with L-DOPA. The effect of sodium status would suggest that the enzyme is regulated by salt intake. No significant effect of L-DOPA and of 5-HTP on corticosterone secretion from tissue preparations was observed, which could indicate that dopamine and 5-HT modulate aldosterone secretion through an action on aldosterone synthase. The results from this study provide evidence for a role for L-AAAD in converting circulating L-DOPA and 5-HTP to dopamine and 5-HT. This conversion would then act to either inhibit or stimulate aldosterone secretion from the rat zona glomerulosa. In particular the concentrations of 5-HTP measured in rat blood were similar to those levels found

to produce aldosterone secretion both in rat adrenal capsules and rat glomerulosa cells. So in effect a small regulatory system for aldosterone secretion would seem to exist within the adrenal gland of the rat, possibly via a feedback loop mechanism.

The presence of L-AAAD within the ZF of the rat adrenal gland requires further attention. This may be important in corticosterone modulation. Another study would be to look at 5-HT within the ZF, to elucidate if the indole has any effect within this region. The putative presence of the 5-HT transporter within the adrenal cortex has been determined, but as yet no evidence has been shown for the existence of a transporter for dopamine or L-DOPA. Competition experiments using an inhibitor of this system, 3-o-M-DOPA may be able to determine the presence of such a system (Soares-da-Silva *et al.*, 1994). The possibility of changing intracellular pH as a mechanism of regulation of L-AAAD could also be investigated. The use of pH-sensitive fluorescent probes, such as BCECF, on cells taken from rats on differing salt diets would indicate if pH was affected by salt diet. The effect of manipulating the intracellular pH of isolated ZG cells on aldosterone biosynthesis, through the use of such agents such as NH_4Cl and Na^+ proprionate, could then be investigated.

The results of this study suggest that the enzyme L-AAAD, present within the rat adrenal cortex, may form part of a paracrine or autocrine mechansim for the modulation of aldosterone secretion by 5-HT and dopamine.

The role of the 5-HT transporter was studied within the rat adrenal gland. The transporter molecule was visualised throughout the rat adrenal medulla via immunohistochemistry. The 5-HT uptake inhibitors citalopram and DMI were incubated with or without 5-HT in isolated rat zona glomerulosa cells and the resulting aldosterone secretion was measured. Results varied and this was thought to be due to

the collagenase digestion process destroying the transporter, therefore whole capsular tissue was studied in a superfusion system and aldosterone secretion measured. In all cases, both reuptake inhibitors increased the aldosterone output from capsular tissue. The drug of abuse MDMA was also used in this system; this compound also caused an increase in aldosterone secretion. *In Vivo* studies with MDMA produced interesting results with an increase in plasma aldosterone and PRA.

From this study, evidence has been found for the existence of the 5-HT transporter molecule within the rat adrenal gland. MDMA may induce a rise in aldosterone concentration within the plasma by a direct action on the 5-HT transporter within the adrenal gland.

In conclusion, the 5-HT transporter has been localised within the rat adrenal medulla. Staining was also apparent in areas of the cortex associated with the vasculature. This may be due to trapped platelets, or to the presence of chromaffin cells within the cortex (Bornstein *et al.*, 1992). Schroeter *et al.*, (1997) reported the presence of the 5-HT transporter within the rat adrenal medulla and found very intense immunostaining for the transporter, within the chromaffin granules, at the border between the cortex and the medulla. They postulate that these cells are readily positioned to regulate corticomedullary reactions. Pharmacological studies with the 5-HT uptake inhibitors, citalopram and DMI, and the drug of abuse MDMA would support a role of the 5-HT transporter within the cortex, although this needs further clarification. The drug of abuse, MDMA, produced elevations in aldosterone and PRA levels in the plasma of rats. These may cause the side-effects seen with ingestion of MDMA or may be secondary to acute hypovolaemia and hyponatraemia. Further studies will include the uptake of tritiated 5-HT into isolated ZG cells and whole capsular tissue and more

extensive immunohistochemical studies and physiological studies within the whole rat to elucidate a role for the transporter within the gland and in depression

This thesis has provided strong evidence for a physiological role of 5-HT in the control of aldosterone secretion in the rat. 5-HT can be produced locally within the adrenal cortex and induce aldosterone secretion and this action may be switched on in cases of low sodium status. Further studies are required to fully characterise the 5-HT receptor, to elucidate a role for the transporter within the gland and to understand more fully the importance of 5-HT and indeed dopamine, in the physiology and pathophysiology of the adrenal cortex.

In conclusion, 5-HT acts in a multifunctional capacity to maintain normal mineralocorticoid secretion. 5-HT may play a role in the regulation of glucocorticoid secretion during stress, inflammation and allergic phenomena. The interactions seen between cortical and medullary tissue may provide crosstalk between the immune system and the hypothalamo-pituitary-adrenal axis. 5-HT may also play a role in the physiopathology of aldosterone disorders such as primary hyperaldosteronism. Further investigations are required to clearly establish the physiological role of the amine but these studies provide a small insight into the potentially large and now unfolding area of research into 5-HT and its role in adrenal homeostasis.

And finally, the amine 5-HT is such a diverse agent with many functions, summed up quite aptly by Bean & Funk (1959) :

This man was addicted to moanin';

Confusion, oedema and groanin';

Intestinal rushes, great tri-coloured blushes;

And died from too much serotonin!

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